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200 gm Dextri-Maltose,² 90 gm distilled water, and 20 gm salt mixture³ per day. It supplied approximately 2240 cal. A vitamin mixture,⁴ prepared in 2% acetic acid, supplied per day 1 mg thiamine, 1 mg riboflavin, 1 mg pyridoxine, 1 mg inositol, 1 mg p-aminobenzoic acid, 5 mg calcium pantothenate, 5 mg choline, and 15 mg niacin. The casein also contained 0.09 mg riboflavin per day. Each meal consisted of one-third of the diet and one-third of the vitamin mixture. One cup of black coffee was allowed with each meal. Two drops of percomorph liver oil,⁵ supplying about 5000 USP units of vitamin A and 550 units of vitamin D, and 50 mg ascorbic acid⁴ were given with breakfast. The subjects were maintained on this synthetic diet for 7 weeks.

After a month's respite, the following natural diet was used for a period of 3 weeks: 50 gm egg, 100 gm milk, 50 gm cheese,⁶ 100 gm ground beef chuck, 20 gm cooked bacon, 100 gm boiled potatoes, 100 gm fresh carrots, 100 gm frozen green beans, 30 gm lettuce, 100 gm tomato juice, 100 gm frozen peaches, 150 gm frozen applesauce, 35 gm raisins, 30 gm cornflakes, 30 gm cooked rice, 26 gm Ry-Krisp, 50 gm peanuts, 15 gm mayonnaise, 35 gm butter, 20 gm brown sugar, and 24 gm peppermint candy per day. This diet was calculated to have approximately the same food value as the synthetic diet, but was found on analysis to contain an average of 0.84 mg thiamine, 0.16 mg less than the synthetic diet.

Collection and care of samples. Twenty-four-hour urine specimens were preserved in brown bottles containing enough glacial acetic acid to maintain an acid concentration of 2 to

² Special vitamin-free Dextri-Maltose, supplied through the courtesy of Mead Johnson and Co.

³ Twenty gm of salt mixture contained 1.75 gm CaHPO_4 , 0.80 gm CaCO_3 , 1.33 gm KH_2PO_4 , 4.00 gm KHCO_3 , 1.50 gm KCl , 2.50 gm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 7.00 gm NaCl , 1.00 gm MgCO_3 , 0.10 gm $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 gm $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.005 gm CuSO_4 , 0.005 gm ZnSO_4 , and 0.00015 gm KI .

⁴ B vitamins and ascorbic acid supplied through the courtesy of Hoffmann-La Roche, Inc.

⁵ Abbott.

⁶ Cheese supplied through the courtesy of Kraft Cheese Corp.

3%. Samples were stored under refrigeration, and were analyzed within 72 hours. Feces were marked with carmine. Individual feces specimens were made 10% acid by addition of glacial acetic acid, and preserved under refrigeration in 5-day composites. During 4 days of the study on the natural diet, samples of the foods as eaten were collected at each meal, immediately macerated in a Waring blender, and made to volume. Aliquots were quick frozen for later analysis.

Methods of analysis. The thiamine content of urine, feces, and foods was measured by the modification by Najjar and Ketrone ('44) of the Hennessy thiochrome method ('42). Feces were examined for both "free" and "total" thiamine, i.e., before and after samples had been incubated with Mylase P.¹

RESULTS AND DISCUSSION

Urinary excretion of thiamine. The values for the average daily intake and excretion of thiamine by the three subjects are given by 5-day periods, table 1. The daily urinary excretion values on the synthetic diet were, for subject A, 116 ± 17.9 μ g, for subject B, 113 ± 20.0 μ g, and for subject C, 147 ± 18.1 μ g. On the natural diet the corresponding values were 90 ± 8.7 μ g, 91 ± 17.5 μ g, and 112 ± 20.3 μ g. The lower values on the natural diet reflect the lower intake of thiamine on this diet (a reduction of 160 μ g per day). The 5-day averages were relatively constant for each individual, but subject C excreted an average of 31 to 34 μ g more than the other subjects while on the synthetic diet, and 21 to 22 μ g more while on the natural diet. Since subject C weighed 10 and 5 pounds more than subjects A and B, respectively, and the food intake of all subjects was the same, this difference in excretion is not related to body weight or food intake.

These urinary excretion values are similar to those reported in the literature for subjects on comparable thiamine intakes. The fact that on the synthetic diet there is no tendency for decreased urinary excretion might indicate that the level of

¹ Wallerstein Laboratories.

TABLE 1
Average daily excretion of thiamine by three women.

PERIOD	SUBJECT									
	A			B			C			
	Urine:		Total	Urine:		Total	Urine:		Feces	Total
	Free	Combined		Free	Combined		Free	Combined	Free	
	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg
Synthetic diet containing 1.00 mg thiamine per day										
1	99	97	158
2	122	18	121	142	15	37	178	13	93	284
3	87	17	107	95	16	35	138	15	49	202
4	107	9	112	109	19	91	135	15	133	283
5	112	19	127	106	10	42	138	2	30	170
6	120	14	70	109	17	29	138	31	77	246
7	127	23	90	106	6	21	148	12	106	266
8	128	28	119	120	20	62	151	9	89	249
9	138	7	43	137	20	63	142	8	73	223
Av.	116	17	99	113	15	48	147	13	81	240 ¹
Natural diet containing 0.84 mg thiamine per day										
1	95	30	268	109	43	292	137	30	386	553
2	91	22	184	99	53	336	117	23	268	408
3	84	12	271	78	47	145	93	30	535	658
4	90	38	229	78	54	122	100	55	272	427
Av.	90	25	238	91	49	244	112	35	365	512

¹ Period 1 omitted in computing total excretions.

1.0 mg per day was adequate for maintenance of thiamine needs for these subjects. The decrease in urinary thiamine excretion for subjects B and C on the intake of 0.84 mg might indicate that this level of intake is lower than their customary ingestion of the vitamin. The excretion might have decreased further had the study been continued.

Fecal synthesis and excretion of thiamine. The values for the average daily fecal excretion of thiamine by periods are included in table 1. On the synthetic diet general averages for "free" thiamine for eight 5-day periods were 17, 15, and 13 μ g, values far below those found by Najjar and Holt ('43) in subjects who were free from deficiency symptoms when they were on thiamine-free diets. On the natural diet the average "free" thiamine excretions were increased to 25, 49, and 35 μ g per day. The values for "combined" thiamine (total thiamine minus "free" thiamine) on the diet of natural foods were 2.4, 5.1, and 4.5 times greater than the values on the synthetic diet.

The "combined" thiamine is generally thought to lie within the bacterial cells, and to be unavailable for assimilation. Alexander and Landwehr ('45) contend that no thiamine is absorbed from the colon, and consequently that the free thiamine as well as the combined is unavailable. This conclusion is based on a comparison of the urinary and fecal thiamine excretion of one subject on 2 successive days, the second day following a retention enema containing added thiamine and coenzyme. From the daily variations in urinary thiamine excretion found in the subjects of the present study and the difficulty in marking 24-hour stools, it seems unjustifiable to base such a conclusion on the evidence of one set of tests on one subject.

The fact that less thiamine was excreted in the feces on the synthetic diet than on the natural diet might indicate that the conditions on the synthetic diet were less favorable for bacterial synthesis of thiamine than those on the natural diet. The facts that the urinary thiamine excretions on the natural diet reflected the lowered thiamine intake, and that the in-

creases in fecal excretion of free thiamine were small, might indicate that even on the natural diet fecal synthesis of thiamine is not an important factor in the thiamine economy of these three subjects.

Significance of the results in relation to human requirement for thiamine. In recent reviews of the experimental data available as a basis for the determination of the thiamine requirement of man, Melnick ('44) concludes that 0.6 mg per 1000 cal is not an excessive allowance, while Holt ('44) considers that between 0.17 and 0.23 mg per 1000 cal is the basic requirement. These values probably represent the extremes between which the average requirement will be found. In the revised recommended dietary allowances of the National Research Council ('45), the thiamine allowance for women has been set at 1.1 to 1.2 mg.

As a result of recent extensive studies on men having a restricted intake of B vitamins, Keys and associates ('45) conclude that clinical impressions do not give conclusive evidence of thiamine deficiencies. Although urinary thiamine excretions are probably significant, the level of excretion which indicates adequate thiamine metabolism is still questionable. They also question whether the thiamine requirement is actually strictly and linearly proportional to calories, or even to the non-fat calories, and suggest that man may require a constant amount, plus an amount related to total or non-fat calories. From these and other data in the literature (Jansen, '32; Van Veen, '40; Elsom et al., '42) it appears probable that 0.6 to 0.7 mg per day will prevent symptoms of deficiency for at least a month, and that 1.0 mg will allow a small margin of safety in healthy persons for 6 months or perhaps indefinitely.

The thiamine intakes of subjects in the present study (0.84 to 1.00 mg per day, or 0.37 to .45 mg per 1000) lie between the limits suggested above. No symptoms of deficiency were evident on the lower level of intake, but the daily urinary thiamine excretion fell below 100 μ g for two of the subjects. These intakes appear to have been adequate for the periods

during which they were used, and the results of the study support the evidence of other workers that lowering the recommended daily allowance for women to 1.1 or 1.2 mg per day gives an adequate margin of safety under normal conditions.

SUMMARY AND CONCLUSIONS

Three normal women were maintained on a synthetic diet containing 1.00 mg of thiamine per day for 7 weeks. This diet was followed, after a month's respite, by a diet of natural foods containing 0.84 mg of thiamine per day. Excretion of thiamine in the urine was measured daily, and in the feces by 5-day periods. The results may be summarized as follows:

1. Daily urinary thiamine values averaged 116, 113, and 147 μ g on the synthetic diet, and 90, 91, and 112 μ g on the natural diet.

2. The average values for daily excretion of "free" thiamine in the feces were 17, 15, and 13 μ g on the synthetic diet, and 25, 49, and 35 μ g on the natural diet.

3. "Combined" thiamine of the feces was 2.4, 5.1, and 4.5 times higher on the natural than on the synthetic diet, suggesting that the synthetic diet was less favorable for bacterial synthesis of thiamine than the natural diet.

4. The facts that urinary thiamine excretions on the natural diet reflected the lowered thiamine intake and that increases in fecal excretion of "free" thiamine were small, might indicate that fecal synthesis of thiamine was not an important factor in the thiamine economy of these three subjects.

5. The excretions of thiamine by these subjects and their apparent well-being on thiamine intakes of 0.84 to 1.00 mg support the lowering of the recommended daily allowance for women to 1.1 or 1.2 mg per day.

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A COMPARISON OF RIBOFLAVIN SYNTHESIS AND EXCRETION IN HUMAN SUBJECTS ON SYNTHETIC AND NATURAL DIETS

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The observation by Najjar and Holt ('43) of the biosynthesis of thiamine in man, stimulated interest in fecal synthesis as a source of other vitamins of the B complex. In 1944 Najjar and coworkers reported synthesis of riboflavin in man. Data are presented in the present study on synthesis and excretion of riboflavin by three women on the synthetic diet of Najjar and Holt ('43) and on a diet of natural foods of similar nutritive value. Details concerning the subjects, diets, and collection and preservation of samples are given in the report by Hathaway and Strom ('46).

The riboflavin content of the samples was determined by the method of Najjar ('41).

RESULTS AND DISCUSSION

It is difficult to plan a diet adequate in other dietary essentials which contains as little as 1.0 mg of riboflavin. In this study the riboflavin content of the natural diet proved on analysis to be higher than had been calculated. The synthetic diet contained 1.09 mg per day of riboflavin, or 0.48 mg per 1000 cal, the natural diet 1.33 mg per day or 0.59 mg per 1000 cal. These differences in riboflavin intake complicate the comparison of the urinary excretions of the subjects on the two diets, but probably do not alter the fecal excretions.

Davis ('45) found fecal excretions in five subjects unaltered by variations in riboflavin intake.

Urinary excretion of riboflavin. The average daily intake and excretion of riboflavin by these three subjects are reported by 5-day periods in table 1.

On the synthetic diet the average urinary excretion of riboflavin decreased markedly until the end of the third period, suggesting that the normal riboflavin intake of these subjects had been greater than the 1.09 mg in the synthetic diet. By period 4 the values for subjects B and C had approached a plateau, but single high values (over 230 μ g) during periods 4 and 5 held these 5-day averages for subject A above her average for the last 6 periods. Daily urinary excretions for periods 4 through 9 were 165 ± 25.9 μ g, 152 ± 19.2 μ g, and 161 ± 24.1 μ g for subjects A, B, and C, respectively. The urinary riboflavin excretions were greater on the natural diet than on the synthetic diet but by period 2 had reached a plateau for subjects A and C. They were still high for subject B. Excretions during the last three periods were, for subject A, 174 ± 35.4 μ g, for subject B, 229 ± 48.7 μ g, and for subject C, 210 ± 35.6 μ g.

It is interesting to compare these data with those from a preliminary experiment planned primarily to study thiamine metabolism. Subject B took part in this study, in which a different diet of natural foods was used (Giff, '44). Her riboflavin intake was 1.40 mg per day, or 0.54 mg per 1000 cal. During the first 2 weeks daily determinations of urinary riboflavin were made, and for the last 4½ weeks, analyses were made of two 3-day aliquots per week. Values for these last nine determinations were 252 ± 40.6 μ g. Corresponding values for a second woman on the same level of intake were 269 ± 39.1 μ g. Two men also acted as subjects for this study. Their riboflavin intakes were higher than for the women, one subject on an intake of 1.49 mg or 0.48 mg/1000 cal, excreted 266 ± 35.5 μ g, and the other on an intake of 1.56 mg or 0.44 mg/1000 cal, excreted 382 ± 31.6 μ g. The riboflavin ex-

TABLE 1
Average daily excretion of riboflavin.

PERIOD	SUBJECT										
	A			B			C				
	Feces			Feces			Feces				
	Urine, Free	Free	Total	Urine, Free	Free	Total	Urine, Free	Free	Combined	Total	
	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg
	Synthetic diet containing 1.09 mg riboflavin per day										
1	269			311			478				
2	207	317	754	308	123	44	375	237	192	804	
3	203	332	723	224	178	130	239	300	187	720	
4	196	298	609	169	218	99	179	387	194	750	
5	177	325	98	144	125	40	182	113	59	354	
6	155	153	59	146	154	76	170	288	166	654	
7	157	273	62	146	58	22	146	280	108	600	
8	144			153			159				
9	162	156	97	152	222	92	132	165	83	385	
Av. 4-9	165	241	86	152	155	66	161	218	133	543 ¹	
	Natural diet containing 1.33 mg riboflavin per day										
1	197	616	530	321	759	310	333	697	579	1609	
2	174	642	406	273	643	199	234	510	464	1208	
3	161	584	646	194	360	297	167	997	949	1813	
4	187	872	494	219	695	307	227	718	589	1534	
Av. 2-4	174	699	515	229	566	268	210	742	567	1518	

¹ Period 8 omitted in computing average excretions, periods 4 to 9.

cretions on these higher intakes showed more relation to total intake than to the intake per 1000 cal.

On diets of widely different composition but with a relatively narrow range of riboflavin content (1.0 ± 0.1 mg), the following values have been reported for 24-hour urinary excretions of riboflavin: Williams et al. ('43) 48 to 212 μ g; Foltz et al. ('44) 120 to 310 μ g; Keys et al. ('45) 113 to 155 μ g; Davis ('45) 92 to 210 μ g, averaging 150 μ g. The riboflavin excretions for each subject averaged 100 μ g or more. In the present study, during the last six 5-day periods on the riboflavin intake of 1.09 mg, the urinary excretion values varied from 108 to 240 μ g, averaging 152 to 165 μ g. These values are in good agreement with the ones reported above.

On the riboflavin intake of 1.33 mg per day, the 24-hour urinary excretions averaged 174 to 229 for the last three 5-day periods. One subject in the Williams et al. study ('43) with intakes at this level, excreted 138 to 156 μ g in 3 tests. Davis ('45) reports excretion values for 4 women at about this level of intake, of from 188 to 486 μ g, averaging 274 μ g for 5 weeks. Thus the excretions at this level are greater than at 1.0 mg, and averages vary more than at the lower level of intake.

Table 2 includes riboflavin excretion values for 1-hour fasting urine specimens collected during 15 days of the study on a daily intake of 1.09 mg of riboflavin. The values varied from 3.7 to 10.9 μ g per hour, with average values for the three subjects of 5.9, 7.1, and 7.8 μ g. These correspond to the ones reported for Davis' subjects ('45) on intakes of approximately 0.60 mg rather than those on intakes of 1.02 mg. They are in the range reported by Najjar et al. ('44) for 12 young boys on diets containing only 70 to 90 μ g per day.

Evidence is presented by Feder, Lewis and Alden ('44) that the riboflavin excretion per milliliter of urine is a more constant value than the excretion per day or per hour. Contrary evidence is presented in table 2. The relationship of the urinary riboflavin excretion to the urinary volume, both on 24-hour and on 1-hour collections, is given for 15 successive

days for the three subjects. The above authors also state that for the most part the micrograms of riboflavin per milliliter in fasting morning specimens agreed well with the corresponding figures for 23-hour samples. This was true only for the specimens for subject A on November 30th. On December 14th and 16th, when hourly urine samples for 6 hours were desired for ascorbic acid excretion tests (Delaney, '45), it was necessary for subjects to drink more water than on the 13th and 15th. Comparison of the riboflavin excretion values for these 4 days shows much closer agreement between the micrograms per 24 hours than per milliliter. The results of the present study do not support the suggestion that urinary riboflavin excretion is related to urinary volume.

Fecal synthesis and excretion of riboflavin. Values for the average daily fecal excretion of riboflavin are included in table 1. Considerable amounts of "free" riboflavin were found in the feces of all three subjects on the synthetic diet. The amounts on the natural diet were 2.9, 3.7, and 3.0 times greater than those found on the synthetic diet. The "combined" riboflavin (that found within the bacterial cells, and freed for analysis by action of Mylase P) showed even greater increase on the natural diet, undoubtedly due to an increased bacterial content of the feces. The total riboflavin excretions on the natural diet were 2.8 times greater than those on the synthetic diet for all three subjects.

The total amounts of riboflavin excreted by these three subjects on the synthetic diet approximate half the intake, and consequently indicate nothing concerning riboflavin synthesis. On the natural diet, however, the average total excretion of riboflavin for the last 15 days was greater than the intake for subjects A and C, and only 268 μ g less than the intake for subject B. The fecal riboflavin for subject B was considerably below that for subjects A and C throughout the study.

In the studies by Najjar et al. ('44), the fecal riboflavin excretions by boys on synthetic diets containing only 70 to 90 μ g of riboflavin per day, varied from 80 to 1276 μ g, but

the majority of the values were between 200 and 600 μg per day. In the present study the total fecal excretions on the synthetic diet varied between 80 and 571 μg , but were generally between 200 and 500 μg . The fecal excretions were comparable in the two studies.

On natural diets containing from 0.28 to 0.66 mg riboflavin per 1000 cal, or absolute values of about 500 to 1400 μg per day, excretion values varying from 399 to 872 μg per day were found by Davis ('45) in five women subjects. Values for a given individual seemed to be independent of the riboflavin content of the diet. The three subjects on the present study excreted 657 to 1646 μg per day in the feces while they were on the natural diet, with 10 of the 12 values between 840 and 1375 μg . In all of these studies individual differences in fecal riboflavin excretion were noted. Differences in the composition of the diets in these studies appear to be more important in determining fecal riboflavin excretion than are riboflavin intakes.

The evidence presented in this report leads to the conclusion that the conditions when the natural diet was used were more favorable for bacterial synthesis of riboflavin than those when the synthetic diet was ingested. What effect this increased synthesis may have had on the absorption and urinary excretion of riboflavin is not clear, since the riboflavin intakes were higher on the diet producing the larger amount of riboflavin synthesis.

Value of riboflavin excretion studies as a measure of requirement. Studies have been reported from several laboratories in which subjects on very low riboflavin intakes have excreted considerable amounts of riboflavin, and have exhibited none of the expected symptoms of riboflavin deficiency (Najjar and associates, '44; Hagedorn et al., '45; and Keys et al., '45). Work of Michelson, Doeden and Keys ('45) has demonstrated that urinary riboflavin excretion is not related to riboflavin intake only, but may be significantly altered by factors such as complete starvation, hard physical work, acute

athiaminosis, and bed rest. The true significance of urinary excretion of riboflavin has not yet been established, and all factors affecting it are not known. The whole picture is complicated by the fact that the specificity of the formerly accepted symptoms of deficiency, corneal vascularization and cheilosis, has been questioned.

Extensive evidence has been given in papers mentioned above of total excretions of riboflavin far in excess of the intakes over considerable periods of time. The evidence of large amounts of "free" riboflavin in the feces suggests that this might be a source of the urinary riboflavin, but no means of measuring conditions leading to its absorption have been suggested. May there be appreciable absorption of fecal riboflavin when the riboflavin concentration in the body is low, and less absorption at other times? Such a condition has been shown to exist in regard to iron (Ross and Chapin, '41). Evidence has been presented (Najjar et al., '44) that riboflavin is not excreted into the intestinal tract.

Excretion studies add to our information concerning riboflavin metabolism, but do not give us the answer to the question of requirement.

SUMMARY AND CONCLUSIONS

Three normal women were maintained for 7 weeks on a synthetic diet containing 1.09 mg of riboflavin per day. This diet was followed, after a month's respite, by a diet of natural foods containing 1.33 mg riboflavin per day. Throughout the study 24-hour urinary excretions of riboflavin were determined, and during 2 weeks of the time on the synthetic diet, 1-hour fasting excretions were also measured. Fecal riboflavin excretions were measured by 5-day periods. The results may be summarized as follows:

1. Daily urinary riboflavin values for the three subjects averaged 165, 152, and 161 μ g on the synthetic diet, and 174, 229, and 210 μ g on the diet of natural foods.

2. The 1-hour fasting urinary excretions of riboflavin varied from 3.7 to 10.9 μg , with average values for the three subjects of 5.9, 7.1 and 7.8 μg .

3. Urinary riboflavin excretion showed no relation to urinary volume.

4. Fecal riboflavin excretions on the diet of natural foods were 3.7 to 3.8 times greater than those obtained on the synthetic diet.

5. On the diet of natural foods, total riboflavin excretions were 2.8 times greater than on the synthetic diet for all three subjects, and for two subjects exceeded the intake.

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THE DEVELOPMENT AND DEMONSTRATION OF CORNEAL VASCULARIZATION IN RATS DEFICIENT IN VITAMIN A AND IN RIBOFLAVIN

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TWO PLATES (TWELVE FIGURES)

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The invasion of the cornea by capillaries has often been considered to be a symptom of nutritional deficiency disease. This vascularization of the cornea has been observed in rats deficient in riboflavin (Bessey and Wolbach, '39), vitamin A (Wolbach and Howe, '25), tryptophane, lysine, zinc or sodium (see the review of Dann and Darby, '45). We have recently reported a similar invasion to take place in rats subjected to a methionine-deficient diet or to a diet devoid of protein (Sydenstricker, Hall, Hock and Pund, '46). In man corneal vascularization has been observed to result from riboflavin deficiency (Sydenstricker, Sebrell, Cleckley and Kruse, '40). This responds to riboflavin therapy (Sydenstricker, Kelly and Weaver, '41). In both rats (György, '42) and man (Lyle, Macrae and Gardiner, '44) there seem to be unknown nutritional factors of importance in preventing vascularization of the cornea. It is of interest that thallium poisoning (see Buschke, '43) or a high tyrosine diet (Hueper and Martin, '43) can result in vascularization of the cornea.

Physical and chemical trauma and infections may also result in corneal vascularization (Duke-Elder, '44). Lowry and Bessey ('45) have recently investigated the effects of light, trauma, riboflavin and ariboflavinosis on the production of corneal vascularity. "Brilliant continuous illumination with incandescent lamps did not augment the changes in the cornea and conjunctiva resulting from riboflavin deficiency" nor did the illumination affect the riboflavin content of the cornea (Bessey and Lowry, '44). Corneal changes in ariboflavinosis appeared when the riboflavin content of the cornea had fallen to less than half of normal. This would seem to indicate that the corneal changes were evidence of rather extreme depletion of riboflavin. The administration of extra riboflavin to normal rats had no detectable influence on the development of "spontaneous" corneal vascularity (Lowry and Bessey, '45). The administration of riboflavin has also been shown to have no effect on the corneal vascularization resulting from tryptophane deficiency (Albanese, '45) or that resulting from deprivation of protein (Sydenstricker, Hall, Hock and Pund, '46).

All in all, the mechanism of corneal vascularization, as well as some of the causes of this condition, seem to be obscure.

We have undertaken to investigate possible causes for the development of corneal vascularization and, at the same time, to try to accumulate information which might lead to a better understanding of this phenomenon. The present paper is concerned with a description of the methods and techniques which we have adopted, with the variation seen in the normal rat cornea, and with our observations on the corneal changes resulting from riboflavin and vitamin A deficiencies.

METHODS

In our investigation of the normal variation in the cornea of the rat with the bionicroscope, we have examined the eyes of some 500 of our Wistar strain rats. All of these rats up to the time of examination were on a diet of commercial dog food.¹ In

¹ Purina Dog Chow Checkers.

addition, they received milk daily and ample amounts of lettuce and carrots at least three times a week. Of the 500 rats used, 78 were adult rats when first examined and 20 others were followed from weaning to adulthood with periodic biomicroscopic examination. Two hundred and sixty-five rats were from 21 to 40 days of age when examined and the remainder of the rats were of ages varying from 40 to 120 days.

Nine rats from three litters and later two litters of seven and of six rats, respectively, were placed on a riboflavin-deficient diet when from 25 to 29 days of age. Eight rats from the first three litters and twelve other rats from different litters of the same strain were placed on the control diet. The experiment was further controlled by producing regression of corneal changes with the administration of riboflavin to some of the rats. The riboflavin-deficient diet consisted of vitamin free casein² 20 gm, salt mixture (as used by McKibben, Madden, Black, and Elvehjem, '39) 4 gm, cod liver oil (U.S.P.) 2 gm, cottonseed oil 2 gm, sucrose 71.7 gm, choline chloride 250 mg, thiamine 10 mg, pyridoxine 10 mg and calcium pantothenate 20 mg. The last mentioned group of six rats on the riboflavin-deficient diet received a supplement of small amounts of riboflavin as follows: At the end of the second week on the diet, a supplement to supply 3 μ g of riboflavin per day was started. This was administered in a 20% solution of ethyl alcohol, twice a week. Since some of the animals had not developed corneal vessels, 50 days later this was cut to 2.4 μ g per day. The control diet was similar to the riboflavin-deficient diet except that it contained 1.6 mg of riboflavin per 100 gm of diet.

In order to follow the corneal changes resulting from vitamin A deficiency, a litter of six Wistar strain rats was placed on the vitamin A deficient diet when 25 days of age. Later, another litter of six was placed on the same diet when 41 days of age and a third litter of five at 36 days of age. The diet which they received consisted of vitamin free casein² 20 gm, salt mixture (as used by McKibben, Madden, Black and

² Labco.

Elvehjem, '39) 4 gm, suerose 73.8 gm, eottonseed oil 2 gm, choline 200 mg, calcium pantothenate 2 mg, riboflavin 1.6 mg, thiamine 4 mg and pyridoxine 4 mg. An equal number of control rats of the same strain, but of different litters, were placed on the same diet as above, except that 2 gm of the suerose had been replaced with cod liver oil, U.S.P.

All the rats on deficient diets were kept in wire cages with screen bottoms, one or two rats to a cage, with food and water supplied ad libitum. The rats were weighed twice a week.

Animals with typical corneal changes for histological studies or injection and subsequent photographing of the cornea were selected with the use of a Bausch and Lomb "Universal slit lamp." For biomicroscopic examination of rat eyes, the slit lamp assembly was equipped with a viewing platform similar to that pictured by Buschke ('43). Berliner ('43) has described the technique for the use of the biomicroscope, which we have used in looking for corneal changes. The eyes of all rats on deficient diets were examined with the biomicroscope at least once a week and usually, during periods when eye changes were occurring, examinations were made daily or bidaily. In describing corneal changes we limit our use of the term "corneal vascularization" to the situation where vessels have penetrated the cornea beyond the limits of variation found in the normal rat.

In order to demonstrate the vascular patterns in corneal vascularization a method similar to that of Bessey and Wolbach ('39) has been used here (Cochran, DeVaughn and Allen, '42) where, after India ink injection of the vessels, a photomicrograph was made of a flat mount of the cornea. For our present studies we have developed a method for photographing the intact cornea which is somewhat like the technique mentioned by Eckardt and Johnson ('39). A description of the technique we use follows:

In preparation for making photographs of the intact cornea, the rats were first killed by the inhalation of chloroform. The anterior chest wall was then cut away and the internal mammary arteries and the descending aorta were clamped with haemostats. A 20-gauge

hypodermic needle (the point of which had been partly ground off), was passed through the left ventricle and into the ascending aorta where it was secured with a silk ligature. Approximately 5 ml of Higgin's India ink was injected slowly, after which the head was removed and fixed in 1:10 formalin.

After sufficient time had elapsed for thorough fixation, the eye was removed by gross dissection. Then, under a binocular dissecting microscope, the conjunctiva was trimmed at the line of reflection at the fornix and all connective tissue and muscle attachments posterior to this line were dissected off. The posterior portion of the eye was next removed by making a circular incision through the sclera and retina just posterior to the ciliary body. This was followed by the removal of the lens. The ciliary body with the iris attached was freed from its attachment to the sclera and removed. The remaining specimen thus consisted of the cornea, the sclero-corneal junction and a narrow rim of sclera. In order to prevent drying, all of the dissection was done with the specimen partially immersed.

The specimen was photographed with the use of a Leitz microscope and a Makam photomicrographic camera (1 \times). A no. 2 photoflood bulb was used for direct substage illumination, with the top lens removed from the Abbé condensor. A Micro Summar photo-objective with focal length of 40 mm was used at f. 24 with a tube length of 120 mm and 6 \times eyepiece. An adapter was devised which made it possible to use Eastman lantern slide plates (medium) in the camera.

In general, for the investigation of histological changes in the cornea, standard techniques were employed which need not be described here. A description of the histological changes in the rat corneas which resulted from the nutritional deficiencies will be described in a later paper.

RESULTS AND DISCUSSION

The cornea of the normal young rat is characteristically clear and limpid. With age there occurs a slight thickening accompanied by some loss of clarity. Also with age there seems to occur a widening of the vascular limbic area and by the time the rat reached adulthood an occasional capillary loop may appear to extend into the edge of the cornea as much as a tenth of the way to the center.

Figures 1 and 2 which are injected corneas from two rats 6 months of age show the degree of vascularity ordinarily

encountered in the limbus of the normal rat's eye. On examination of the limbic area in these two pictures, it is to be noted that there are variations in the width of the limbus due to unequal extension of the vascular transparent scleral margin onto the cornea. These pictures show the approximate limits of normal variation as commonly encountered, so only when corneal capillaries extend beyond the scleral margin as shown here and into the cornea do we apply the term "corneal vascularization." As previously mentioned, the vascular area at the sclero-corneal junction is likely to be narrower in younger rats.

Buschke ('43) states that "recent observations with the slit lamp on a highly inbred strain of rats suggest that vascularization of the cornea occurs also in certain strains of rat as a genotypical trait when the animals are receiving normal diets." Figures 3 and 4 show the opposite sides of a cornea from one of the four animals having apparent spontaneous corneal vascularization, which we have observed among the 500 animals of all ages examined. In these animals the vessels were few and scattered and in marked contrast to the heavy vascularization resulting from most of the types of nutritional deficiencies which produce vascularization, or to the vascularization accompanying an inflammatory reaction. No signs of inflammation or injury to the cornea were seen with the biomicroscope and the vessels were observed to be more or less bilaterally symmetrical. It seems unlikely that the vessels were due to trauma or that they resulted from nutritional deficiency. All four rats in which the vascularization was observed, since weaning had been on a diet of commercial dog food¹ supplemented at least three times a week with milk, lettuce and carrots. Lowry and Bessey ('45) found that this type of corneal vascularization did not regress on the administration of riboflavin. The injected cornea shown in figures 3 and 4, which was from a rat 60 days of age, we consider to be a more or less typical instance of this type of "spontaneous" corneal vascularization.

In riboflavin deficiency the first ocular changes are conjunctival edema and congestion and about a week later marked congestion of the limbic vessels and occasionally faint nebulæ in the cornea. Soon thereafter capillary "sprouts" of the terminal loop type may be seen to extend a short distance into the cornea, usually less than $\frac{1}{4}$ the radius of the cornea.

A few days later intense edema and opacity of the cornea develop so that it is not possible to determine the extent of the vascular invasion. After 7 to 14 days the edema and opacity subside and in the great majority of rats, the cornea is seen to be extensively vascularized, often with capillaries anastomosing across the center. The vascular pattern is apt to be of the terminal loop type although some rats show dendritic patterns. Some rats show cataracts at the time that resolution of corneal opacity occurs; others develop it from 7 to 14 days later. Photophobia is usually obvious from the time that the first ocular changes can be seen.

In about 60% of the rats the first visible invading capillaries seem to stem from the circumferential artery in the superior nasal quadrant; later invasion occurs from the entire complex of circumferential arteries. In about 20% the first invasion is from the "feeder" arteries which rather regularly enter the limbus immediately above the pupil at "12 o'clock" on the circle of the cornea. In about another 20% primary invasion is from the superior temporal quadrant. In general, our observations confirm those of Bessey and Wolbach ('39) with the exception that with frequent biomicroscopic examination of the eyes of the deficient rats, we have observed a transient opacity of the cornea which occurs early in the invasion of the cornea by capillaries. The cause of this opacity will be discussed in a later paper.

At the time of death four of nine in the first group of riboflavin-deficient rats showed minor corneal changes. Two had early corneal vascularization. These rats were 29 days of age at the time they were started on the deficient diet. Of the litter of seven rats which were placed on the riboflavin-deficient diet when 25 days of age, only one showed corneal

changes. This rat at death had numerous vessels extending to a length of more than a fourth of the distance to the center of the cornea. The third group of six rats which were 29 days of age when placed on the riboflavin-deficient diet, all developed extensive corneal vascularization and the typical corneal changes described above. Anterior and oblique views of injected corneas from two of these rats are shown in figures 5, 6, 7 and 8. One rat in the group developed a typical riboflavin cataract. These rats were the ones which received the supplement of small amounts of riboflavin as previously described. None of the control rats showed significant corneal changes on biomicroscopic examination. The riboflavin-deficient diet used would appear to be very low in riboflavin; in fact, the only constituent of the diet containing appreciable amounts of this vitamin is probably the Labco casein. According to Cannon, Boutwell and Elvehjem ('45) samples of this casein preparation were found to contain 0.120 mg of riboflavin per 100 gm of casein. The rats on the riboflavin-deficient diets during the course of the experiment consumed decreasing amounts of the diet starting at about 10 gm of diet per day. This would supply through the casein portion of the diet a maximum of 2.4 μ g of riboflavin per rat per day. It appears that in order for the typical corneal changes of riboflavin deficiency to develop, the rats should receive sufficient riboflavin to survive until the corneal changes appear. The rats in the first two groups died before sufficient time had elapsed for the corneal changes to develop, or died just as these changes were first observed.

In vitamin A deficiency as in the ocular manifestations of other deficiency diseases, conjunctival edema and congestion are apt to precede changes in the cornea. Later xerophthalmia develops. The cornea loses its luster, the surface appears slightly opaque and granular and masses of desquamated epithelium from the palpebral conjunctiva may be seen adherent to the cornea. A few days later (3 to 7) there is general thickening and slight diffuse opacity of the cornea and invading capillaries may be seen originating from the same portions

of the circumferential arteries that have been noted in the description of riboflavin deficiency. From the first the invading capillaries in vitamin A deficiency are apt to be of the dendritic type and frequently they tend to form a dense collar of vascularity extending from $\frac{1}{2}$ to $\frac{3}{4}$ the radius of the cornea. More rarely the invading capillaries are of the terminal loop type and extend entirely across the cornea, anastomosing with loops from the opposite side. After the establishment of a dense corneal plexus, edema and leukocytic infiltration of the cornea are apt to progress to the point where the vessels may no longer be seen with the slit-lamp. As in riboflavin deficiency, evidences of photophobia are commonly present from the time that conjunctival changes are visible. Our observations of the effects of vitamin A deficiency on the cornea of the rat in general confirm the observations of Wolbach and Howe ('25) and of Bessey and Wolbach ('39).

Of the litter of six rats which were placed on the vitamin A-deficient diet when 25 days of age, ocular changes were observed in only one rat. A few days before this rat died, it developed a dense corneal opacity and began to show signs of xerophthalmia. The rats of this litter died on the average in 54 days after being placed on the diet. On a previous occasion we had placed a litter of four rats on a vitamin A-deficient diet when 22 days of age, all of whom died without developing xerophthalmia or corneal vascularization.

Of the litter of six rats placed on the vitamin A-deficient diet at 41 days of age, five showed typical ocular symptoms of vitamin A deficiency as described above beginning about 70 days after being placed on the diet. Figures 9, 10, 11 and 12 are pictures of injected corneas from three of these rats. The cornea shown in figure 9, at the time the animal was killed, showed vascularization only in the superior nasal quadrant, while figures 10 and 11 show the further development of vascularization.

Two rats from the third litter of five which were 36 days of age when placed on the diet, developed early xerophthalmia with a mild degree of corneal vascularization before they died.

The other three rats showed no ocular changes. The rats from this litter lived an average of 78 days after being placed on the diet. None of the rats on the control diet showed any significant eye changes on biomicroscopic examination.

The vitamin A-deficient diet was probably very low in vitamin A. The cottonseed oil used gave no test for carotene with antimony trichloride reagent and probably none of the other constituents contained very appreciable amounts of the vitamin. While it is difficult to draw any very adequate conclusions from four groups of rats, it would seem that the younger rats did not live long enough on the deficient diet to develop the characteristic corneal lesions. Only the rats in the oldest litter which were 41 days of age when placed on the diet, lived to show all the typical corneal changes of vitamin A deficiency. We had observed that with our rats on diets deficient in protein, lysine, or methionine, the age of the rat and the degree of deficiency of the diet were of importance in determining whether corneal vascularization appeared and relatively how soon it developed (Sydenstricker, Hall, Hock and Pund, '46). What we have observed with rats deficient in vitamin A and riboflavin is consistent with this finding:

The ocular changes in the riboflavin and vitamin A deficient rats described in this paper are typical of what we have observed in the examination at various times of a considerable number of rats which were suffering from one or the other of these deficiencies.

On biomicroscopic examination, it was observed that as corneal vascularization began, capillaries usually first invaded the cornea in the superior nasal quadrant. As vascularization progressed, the vessels in this quadrant usually continued to exceed in length the vessels in other quadrants. Occasionally the vessels from two quadrants would exceed in length the vessels from other quadrants. This seems to hold true in all the types of vascularization due to nutritional deficiencies which we have studied. Figure 9 shows the margin of the superior nasal quadrant of the cornea of a vitamin A-deficient rat in which vascularization has proceeded far into the cornea

in this quadrant but has not begun in the other three quadrants. Figures 5 (riboflavin deficiency) and 11 (vitamin A deficiency) show this preponderance in length of the vessels in one quadrant as vascularization proceeds. Figures 10 and 12 (vitamin A deficiency) are pictures of a cornea where the vascularization was greater in two quadrants. Figure 7 (riboflavin deficiency), on the other hand, shows an instance where the development was approximately equal in all quadrants.

In figures 3 and 4 it is to be noted that the capillaries seem to arch over and tend to form loops over a point in about the middle of the picture. On examining photographs of our injected corneal preparations and the preparations themselves, we find that the developing capillaries, as they grow from the circumferential vessel into the cornea, frequently seem to grow from either side of the feeder vessel and to anastomose over the point where the feeder vessel bifurcates into the circumferential vessel. This, in many cases, causes the capillaries to appear to arch over that point.

It would appear that the pattern of vascularization observed when corneal vascularization results from nutritional deficiencies may depend on several factors. The number of capillaries invading the cornea, the distance to which they have penetrated and the extent to which they branch, are probably of importance. It seems that as the capillaries penetrate the cornea their first portions are relatively straight with only fine branches. If the capillaries are numerous and close together, these branches form numerous capillary loops, but if they are fewer in number and farther apart, the dendritic pattern tends to be more apparent. As the vessels converge over the curvature of the cornea, the branches anastomose and the pattern becomes of the capillary loop type. As the vessels grow farther into the cornea the appearance is again dendritic until, by convergence, they once more form capillary loops.

With vitamin A deficiency the numerous capillaries are close together and their branches result in frequent anastomoses forming many capillary loops or a capillary network while in riboflavin deficiency the less numerous capillaries are

farther apart and anastomose less frequently so that at times the dendritic pattern is observed.

SUMMARY

Techniques for preparing and photographing injected corneas of the rat to best demonstrate vascularization are described.

The variation in vascularity at the limbus in rats of various ages has been investigated in some 500 normal rats.

The corneal vascularization resulting from vitamin A and from riboflavin deficiencies has been followed during its development and the type of vascularization which results is described and illustrated.

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PLATE 1

EXPLANATION OF FIGURES

(All $15\times$ reduced approximately one-third.)

- 1 and 2 Injected corneas from normal rats.
- 3 and 4 Opposite sides of an injected cornea showing "spontaneous" corneal vascularization.
- 5 and 6 An injected cornea from a rat deficient in riboflavin.

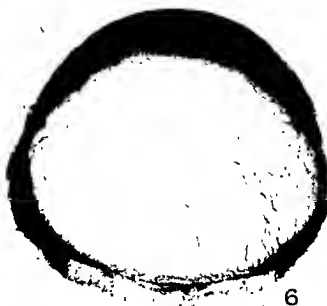
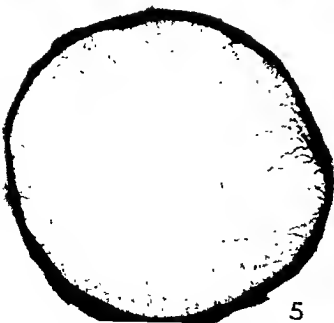
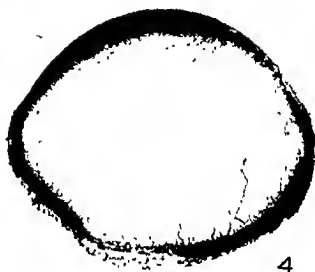
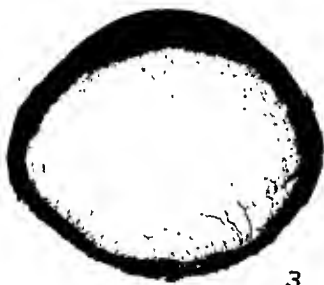
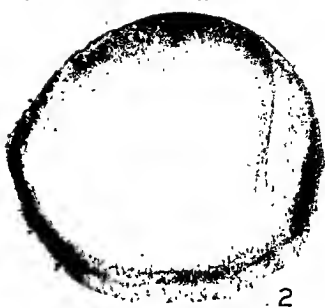


PLATE 2

EXPLANATION OF FIGURES

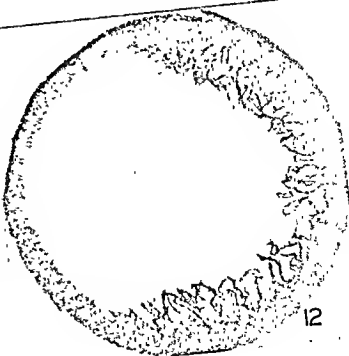
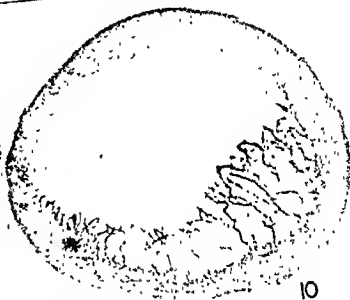
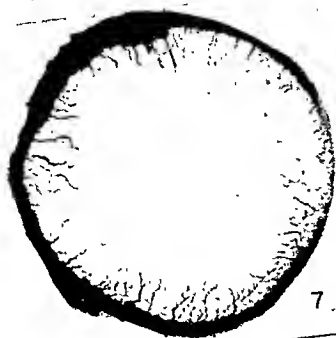
(All 15 \times reduced approximately one-third.)

7 and 8 An injected cornea from a rat deficient in riboflavin.

9, 10 and 11 Injected corneas from three rats deficient in vitamin A.

12 Anterior view of cornea in figure 10.

THE CORNEA IN VITAMIN DEFICIENCIES
LESTER L. DOWLFs AND OTHERS



ATTEMPTS TO PRODUCE A NIACIN DEFICIENCY IN THE RHESUS MONKEY¹

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THREE FIGURES

(Received for publication March 8, 1946)

There are comparatively few reports in the literature dealing with the production of niacin deficiency in laboratory animals fed purified rations. Schaefer et al. ('42) demonstrated a niacin deficiency in dogs which were maintained on a niacin-low purified ration. Briggs et al. ('42) reported that the chick requires niacin for optimal growth and for the prevention of chick "blacktongue" when fed a highly purified ration. Wintrobe et al. ('45) in a recent paper on the niacin requirements of the pig reviewed the literature on niacin deficiency in pigs and concluded that most of the results of the earlier workers were equivocal since the natural rations used were low in proteins and probably some of the B vitamins. They (Wintrobe et al.) made an extensive study of niacin deficiency in pigs, and reported that a dietary source of niacin was not required by pigs which were fed a purified diet containing high amounts of protein. Deficiency symptoms did, however, appear when the protein level was low. Wooley and Sebrell ('45) showed that niacin was a dietary essential for rabbits which were fed a purified ration. Although it has been

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shown (Birch, '39) that the rat does not require niacin when fed a purified ration, Krehl, Teply and Elvehjem ('45) found that a deficiency of this vitamin could be produced in the rat when the purified ration was supplemented with corn grits to the extent of 40%. The production of a niacin deficiency in rhesus monkeys has been reported by Harris ('37) and by Chick and Hume ('20) but the rations employed by both these groups of workers were lacking in adequate protein and B vitamins. In this paper we wish to present the results of attempts to produce a niacin deficiency in monkeys using purified rations alone and purified rations with added corn.

EXPERIMENTAL METHODS

The method of handling the monkeys has been described previously (Waisman et al., '43). The basal ration (M-2) consisting of sucrose 73, Labco casein 18, salts IV 4, cod liver oil 3, and corn oil 2 was fed ad libitum. Adequate amounts of ascorbic acid and all the B vitamins (thiamine, riboflavin, pyridoxine, pantothenic acid, choline, i-inositol, p-aminobenzoic acid and biotin) except niacin were fed daily as a separate supplement. Folic acid was also supplied as a niacin low norite eluate concentrate prepared according to the directions of Krehl et al. ('45) at a level equivalent to 5 gm original solubilized liver (fraction L) per day. In later experiments the synthetic *Lactobacillus casei* factor replaced the norite eluate concentrate and was fed at a level of 100 μ g per day. Microbiological assays (Krehl et al., '43) showed that the basal ration together with the supplement contained less than 50 μ g of niacin per 100 gm.

Studies with purified rations

Four young monkeys (nos. 186, 187, 188 and 189) were placed on the niacin low purified ration. The monkeys continued to grow at a slow rate for several months without showing any deficiency symptoms. At the end of the fifth month, monkey 188 started to lose weight, and niacin therapy at a level of 10 mg per day was instituted. During the next 4

days on this regimen the monkey continued to lose weight rapidly and 25 mg of niacin amide was administered by intraperitoneal injection. The niacin and niacin amide therapy were completely ineffective and the monkey died the next day. Autopsy revealed no gross lesions.

At the end of the sixth month, monkey 189 showed some weight loss. The hemoglobin values also began to decrease and reached a value of 9 gm per 100 ml of blood. Niacin given at a level of 10 mg per day for 1 week proved ineffective in preventing the weight loss, the decrease in hemoglobin content of the blood and the anorexia. The basal ration was

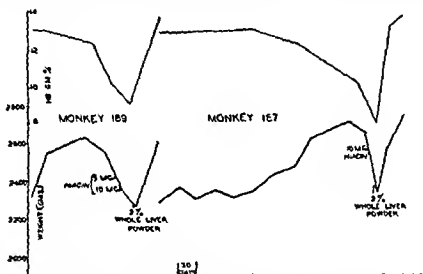


Fig. 1 The response in hemoglobin and weight to niacin and whole liver powder in monkeys which failed on a niacin low purified ration.

supplemented with 3% whole liver powder and fed via stomach tube. Within a week the animal's appetite improved sufficiently to discontinue the tube feeding. Shortly thereafter a sharp increase in weight occurred which was followed by an increase in hemoglobin content of the blood.

Monkey 187 showed a similar history although the animal continued to grow for 14 months before deficiency symptoms appeared. Again niacin was ineffective in correcting the weight loss and drop in hemoglobin, but when the basal ration was supplemented with 3% whole liver powder there was a prompt remission of these symptoms. The data for these animals are summarized in figure 1.

It is apparent from these results that a niacin deficiency can not be readily produced in monkeys fed a purified ration. Since the syndrome precipitated by the niacin low ration was completely reversed by feeding 3% whole liver powder, it is quite probable that a deficiency of the monkey anti-anemia factor existed. These observations are similar in many respects to riboflavin (Cooperman et al., '45b), vitamin B₆ (McCall et al., '46), pantothenic acid (McCall et al., '46), and folic acid (Cooperman et al., '46) deficiencies in the monkey which are complicated by a concomitant deficiency of the monkey anti-anemia factor. There is, however, one distinct difference in the case of niacin, namely, niacin therapy has no beneficial effect upon the deficient animals whereas in the case of riboflavin, vitamin B₆, pantothenic acid, and folic acid there is an initial response to the respective vitamins. The length of time required for the animals to become deficient on the niacin low ration probably depends upon the previous body stores of the monkey anti-anemia factor.

Studies with high corn rations

After having been on the niacin low purified ration for 15 months, monkey 186 still showed no untoward effects. Since Krehl et al. ('45) had demonstrated that rats required niacin when the basal ration was supplemented with 40% corn grits, an attempt was made to produce a niacin deficiency through this means. After 6 weeks on the corn containing ration, monkey 186 began to lose weight rapidly. Twenty-five mg of niacin per day was given for 4 days without any effect on the rate of growth or decrease in hemoglobin. Since Krehl, Teply, Sarma and Elvehjem ('45) had demonstrated that tryptophane could replace niacin as a growth factor for rats when fed the corn grits basal, the monkey was given 300 mg dl-tryptophane per day in addition to the daily supplement of niacin without any beneficial effect. The monkey was then returned to the M-2 basal without the corn grits for 1 week but became progressively worse. The M-2 basal was supplemented with 3 gm of lyophilized liver per day fed via stomach

tube. After 3 days the animal regained its appetite and the tube feeding was discontinued. This treatment was followed by a sharp increase in weight, the animal gained 300 gm in 1 week and the hemoglobin content of the blood increased to 13.5 gm per 100 ml during the same period. The data are summarized in figure 2. The corn grits, in all probability, precipitated a deficiency of the monkey anti-anemia factor since lyophilized liver is an excellent source of this factor (Cooperman, McCall and Elvehjem, '45a).

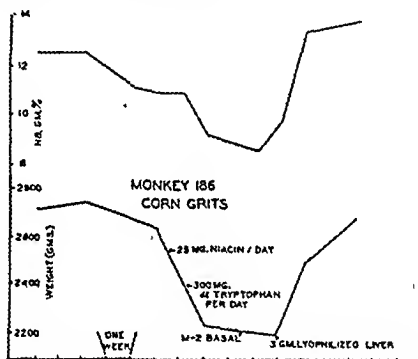


Fig. 2 The effect of feeding niacin, tryptophane, the M-2 basal, and lyophilized liver on the weight and hemoglobin of a monkey which failed on the corn grits basal.

In order to repeat this work with young monkeys, experiments were started in which newly acquired monkeys were placed on the corn grits basal directly. Three young monkeys (nos. 247, 248, and 275) were given the M-2 basal supplemented with 40% corn grits and all the B vitamins except niacin. The hemoglobin content of the blood and differential leucocyte counts were determined regularly on the animals since these are affected in a deficiency of the monkey anti-anemia factor (Cooperman et al., '46; McCall et al., '46). The results with

a typical monkey (no. 248) will be described since all the animals showed a very consistent picture. This animal grew at a very slow rate for 5 weeks at which time growth ceased entirely. The hemoglobin value was 10.6 gm % at this time. The monkey then started to lose weight and at the end of 8 weeks the hemoglobin had decreased to 8.1 gm % and differential leucocyte counts showed a definite reversal in the neutrophile-lymphocyte ratio, the usual syndrome in the monkey anti-anemia factor deficiency (Cooperman et al., '46; McCall et al., '46). At this time 10 mg niacin per day was given for 10 days but this proved inadequate in ameliorating the

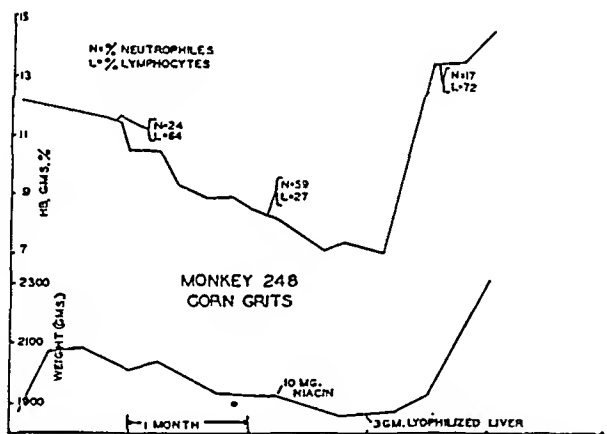


Fig. 3 The effect of niacin and lyophilized liver on the weight, hemoglobin and differential leucocyte counts of a young monkey fed the corn grits basal.

weight loss or the blood dyscrasia. The ration was then supplemented with 3 gm of lyophilized liver per day and within 3 days there was a perceptible change both in the weight and blood picture. At the end of 3 weeks the hemoglobin value reached a level of 14.7 gm % and the neutrophile-lymphocyte reversal was corrected. The data for this animal are summarized in figure 3.

In the case of monkey 247, the corn grits basal was supplemented with both niacin and tryptophane when the weight loss and blood dyscrasia became evident, but this combination also proved ineffective.

Two other young monkeys (nos. 276 and 277) were given the corn grits basal supplemented with 300 mg dl-tryptophane per day and all the B vitamins except niacin. Again growth was poor and the typical blood dyscrasia appeared. Supplementing the ration with 10 mg niacin had no beneficial effect either on the weight or blood condition. One hundred and twenty-five micrograms of crystalline vitamin B₁₂ per day were given to monkey 276 by intramuscular injection for 1 week but this too proved ineffective.

Three monkeys (nos. 295, 296, and 297) were started on the corn grits basal, which was supplemented with all the B vitamins including niacin. A weight plateau appeared at the end of 6 weeks and soon thereafter the typical blood dyscrasia appeared.

Since the typical syndrome of a deficiency of the monkey anti-anemia factor is readily produced when monkeys are fed a 40% corn grits basal, this affords an easy method for the production of assay monkeys. Our previous assay depended upon the weight and hemoglobin response in monkeys which failed to show complete recovery from a riboflavin deficiency after riboflavin therapy (Cooperman, McCall and Elvehjem, '45). Experiments in this laboratory (unpublished data) have shown that both of these types of assay monkeys (corn grits and riboflavin deficient) respond similarly to materials rich in the monkey anti-anemia factor and in no case has any material shown activity in one type without giving a response in the other.

It is interesting to note the different effect the corn grits ration has on the rat and monkey. Whereas in the rat either niacin or tryptophane will correct the deficiency, in the monkey neither tryptophane, niacin nor both are of value in preventing the onset or curing the deficiency.

It will be difficult to ascertain whether the monkey requires niacin until a concentrate of the monkey anti-anemia factor low in niacin is available.

SUMMARY

Monkeys on a purified ration extremely low in niacin develop a deficiency which does not respond to niacin amide therapy. Whole liver powder, a good source of the monkey anti-anemia factor, causes a prompt remission of the deficiency symptoms.

When monkeys are fed a ration containing 40% corn grits, a deficiency characterized by a loss in weight, suboptimal hemoglobin, and a reversed neutrophile-lymphocyte count develops. Neither niacin, tryptophane, nor a combination of the two ameliorate the syndrome. Lyophilized liver or whole liver powder correct the deficiency.

ACKNOWLEDGMENT

We wish to acknowledge our indebtedness to Merck and Company, Rahway, New Jersey, for the crystalline vitamins; to Wilson Laboratories, Chicago, Illinois, for whole liver substance; and to Lederle Laboratories, Inc., Pearl River, New York, for the synthetic *Lactobacillus casei* factor.

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NUTRITIONAL SIGNIFICANCE OF INOSITOL AND BIOTIN FOR THE PIG¹

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TWO FIGURES

(Received for publication January 4, 1946)

McRoberts and Hogan ('44) stated that as yet there is no evidence that the pig requires inositol or biotin for growth. Their work, however, indicated that the young growing pig needs some unrecognized vitamins and that these vitamins are contained in water extracts of liver or yeast.

Cunha, Lindley and Ensminger ('46) showed that the pig fed desiccated egg white developed the following syndrome: alopecia, spasticity of the hind legs, cracks in the feet, and a dermatosis of the skin characterized by dryness, roughness, and a brownish exudate. This syndrome was prevented by intramuscular injection of 100 μ g of biotin per pig daily.

The experiment which is herein reported was undertaken to determine whether the pig needs inositol and biotin in the ration or whether the pig obtains enough of these two vitamins to satisfy its needs through intestinal vitamin synthesis.

EXPERIMENTAL

Six-week old pigs were used in this experiment. These pigs were farrowed by two Chester White gilts which had been

¹ Published as Scientific Paper no. 664, College of Agriculture and Agricultural Experiment Stations, State College of Washington, Pullman.

² This report is from a thesis submitted by D. C. Lindley in partial fulfillment of the requirements for the degree of Master of Science.

bred to a Danish Landrace boar. The dams of these pigs had been fed on a purified ration (sucrose 63.8%, casein 20%, lard 11%, and mineral mix 5.2%) for 52 days prior to parturition and throughout the 6-week lactation period. The purified ration fed to the mothers of these pigs contained only six B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline), and therefore the young pigs were presumably depleted to some degree in biotin and inositol.

The pigs were divided equally as to weight, sex, and age in each lot. The rations fed are shown in tables 1, 2, and 3. The same twelve pigs were used throughout the three phases of this experiment. During the first phase (5-week period) the effect of biotin and inositol on growth of the pig was determined. In the second phase (5-week period), the effect of sulfaguanidine on the biotin and inositol requirements of the pigs was determined. During the third phase (6-week period), the effect of sulfathalidine on the intestinal synthesis of biotin and inositol by the pigs was determined.

The basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger, Cunha and McCulloch ('46). The ration consisted of casein³ 26.1%, sucrose 57.7%, lard 11% and mineral mix 5.2%. Water-soluble vitamins were fed (in mg per kg of live weight daily) as follows: thiamine 0.52, riboflavin, 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat-soluble vitamins were supplied (per pig daily) as follows: vitamin A 5,000 I.U., vitamin D 500 I.U., vitamin E 57 mg, and vitamin K 2 mg.

A modification of the "paired-feeding technique" (Mitchell and Beadles, '30), was used in order to keep the feed intake of all pigs constant. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the smallest

³Casein was prepared by precipitation with HCl and numerous washings with HCl. Casein contained 0.005 μ g of biotin per gram. Biotin determinations on casein were made by Miss M. A. McGregor, Assistant Chemist, Division of Home Economics, according to the method of Shull et al. ('42) as revised by Shull and Peterson ('44).

appetite — with the vitamins and sulfonamides studied being the only variables among the lots of pigs.

The pigs were fed in individual feeding stalls and were kept on raised floors in order to prevent coprophagy. The raised floors were washed twice daily. The type of raised floors used are shown in figures 1 and 2.

To prevent the development of rancidity and consequently the destruction of certain vitamins, the purified ration was kept in a refrigerator and not more than 2 or 3 days feed requirements were mixed at any time.

All vitamins were kept in solution in a refrigerator. They were fed to the pigs every other day. The required amount of vitamins fed was measured in a calibrated pipette and placed on a small amount of the feed on the top of the ration at feeding time. In this manner, struggling with the animals was avoided and complete consumption of the vitamins was obtained.

RESULTS AND DISCUSSION

The data in table 1 show that no beneficial effect on growth or efficiency of feed utilization was obtained when either inositol or biotin was fed in addition to the basal ration. No difference in external appearance could be observed between the control pigs or those fed inositol or biotin. It might be of interest to note the very high efficiency of feed utilization made by the pigs in the three lots (table 1). All pigs made a gain of one pound on less than two pounds of feed.

The pigs in all three lots were observed to chew the board fences occasionally. Fence chewing has been observed previously at this station with pigs fed a similar ration containing 6.2% of oat hulls. Therefore, chewing wood may be due to a deficiency of some factor or factors other than a lack of fiber.

Since the addition of biotin or inositol to the basal ration for a 5-week period was of no benefit, it was assumed that the pig is evidently able, through the action of intestinal bacteria, to synthesize enough of these two vitamins to satisfy its needs. In order to determine whether this was true, sulfa

drugs which have been used to inhibit the growth of intestinal bacteria and hence intestinal vitamin synthesis, were used. The data obtained when sulfonamides were fed are shown in tables 2 and 3.

The addition of sulfaguanidine (sulfanilylguanidine), at the levels fed (table 2), had no effect on the inositol and biotin requirements of the pig. No appreciable beneficial result in growth or external appearance was obtained by the addition of inositol or biotin to the ration of the pigs fed the drug.

TABLE 1

The effect of biotin (B) and inositol (IN.) on growth.

LOT NUMBER	I	II	III
Ration fed	Basal ¹	Basal + IN ²	Basal + B ³
Pig numbers	91, 97, 103, 107	94, 95, 104, 105	92, 93, 101, 106
Initial age, weeks	6	6	6
Number of weeks on trial	5	5	5
Av. initial weight, lbs.	22.4	22.5	22.6
Av. daily gain, lbs.	0.45	0.43	0.41
Av. daily feed consumption, lbs.	0.78	0.78	0.77
Lbs. of feed per lb. of gain	1.73	1.81	1.87

¹ Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline).

² Inositol was fed at level of 100 mg per 100 gm of feed.

³ Biotin was fed at level of 20 μ g per 100 gm of feed.

for a 5-week period. A sub-therapeutic level of the sulfaguanidine was fed during the 5-week experiment. This low level may account for the failure of sulfaguanidine to appreciably inhibit intestinal vitamin synthesis. The drug was not increased to a level greater than 0.75% of the ration toward the latter part of the experiment, since it was thought that sulfaguanidine resistant strains of *Escherichia coli* might be rather numerous by then. Instead it was thought that using another sulfanamide might be more satisfactory in trying to produce a biotin or inositol deficiency. Since the results obtained with sulfaguanidine were not conclusive in determining whether the pig synthesizes biotin and inositol it was

TABLE 2
The effect of sulfaguanidine (SG) on the response of the pig to biotin (B) and inositol (IN).

LOT NUMBER	IA	IB	IIA	IID	IIIA	IIIB
Previous ration (table 1)	Basal	Basal	Basal + IN	Basal + IN	Basal + B	Basal + B
Ration fed	Basal	Basal + liver ¹	Basal + SG ²	Basal + IN + SG ³	Basal + SG ⁴	Basal + B ⁴ + SG
Pig numbers	91 and 107	97 and 103	95 and 105	94 and 104	93 and 101	92 and 106
Initial age, weeks	11	11	11	11	11	11
Weeks on experiment	5	5	5	5	5	5
Av. initial weight, lbs.	36	36.8	37.3	38	37	37
Av. daily gain, lbs.	0.73	0.76	0.71	0.72	0.70	0.79
Av. daily feed consumption, lbs.	1.35	1.35	1.35	1.35	1.35	1.35
Lbs. of feed per lb. of gain	1.85	1.76	1.90	1.83	1.92	1.71

¹ 1-20 liver powder (Wilson) was fed at a level of 2% of total ration.

² Sulfaguanidine was fed at a level equivalent to 0.5% of the ration for the first 4 weeks and at a level equivalent to 0.75% of the ration during the fifth week.

³ Inositol was fed at level of 100 mg per 100 gm of feed.

⁴ Biotin was fed at level of 20 µg per 100 gm of feed.

TABLE 3
The effect of sulfathalidine (ST) on the response of the pig to inositol (IN) and biotin (B).

LOT NUMBER	IAA		IIB		IIAA		IIBB		IIIBB	
	Basal	Basal + liver	Basal + liver	Basal + SG	Basal + IN + SG	Basal + SG	Basal + IN + SG	Basal + SG	Basal + SG	Basal + SG
Previous ration (table 2)	Basal	Basal + liver	Basal + liver	Basal + SG	Basal + IN + SG	Basal + SG	Basal + IN + SG	Basal + SG	Basal + SG	Basal + SG
Ration fed	Basal	Basal + liver + ST ¹	Basal + liver + ST ¹	Basal + SG	Basal + IN + SG	Basal + SG	Basal + IN + SG	Basal + SG	Basal + SG	Basal + SG
Pig numbers	91 and 107	97 and 103	97 and 103	95 and 105	94 and 104	95 and 105	94 and 104	93 and 101	92 and 106	92 and 106
Initial age, weeks	16	16	16	16	16	16	16	16	16	16
Weeks on trial	6	6	6	6	6	6	6	6	6	6
Av. initial weight, lbs.	61.5	63.5	63.5	62	63.8	62	63.8	61.5	64.5	64.5
Av. daily gain, lbs.	0.89	0.96	0.96	0.51	0.98	0.51	0.98	1.04	0.80	0.80
Av. daily feed consumption, lbs.	2.21	2.21	2.21	2.17	2.21	2.17	2.21	2.21	2.21	2.21
Lbs. of feed per lb. of gain	2.49	2.30	2.30	4.24	2.25	4.24	2.25	2.13	2.77	2.77

¹ Liver ("1-20", Wilson) was fed at a level replacing 2.0% of the ration.

² Sulfathalidine was fed at a level replacing 0.5% of the ration.

³ Inositol was fed at level of 100 mg per 100 gm of feed.

⁴ Biotin was fed at level of 20 µg per 100 gm of feed.

decided to try another sulfonamide. Sulfathalidine (phthalyl-sulfathiazole) was decided upon since it had been used successfully by Miller ('45) as an inhibitor of intestinal vitamin synthesis with the rat.

The data obtained when sulfathalidine was added to the basal ration are shown in table 3. Sulfathalidine, at the level fed, proved to be very effective in decreasing intestinal synthesis of vitamins. The addition of sulfathalidine to the basal ration for 6 weeks caused approximately a 40% decrease in daily gains. In addition, the following deficiency symptoms were observed in lot IIAA where sulfathalidine was added to the basal ration. During the third week that sulfathalidine was fed, pig 95 of lot IIAA began to show a hair loss and the appearance of a dark brown exudate on the surface of the skin. The hair loss was first observed on the posterior part of the ham. The hair loss then progressed over the rump, loin, and sides. At the end of the 6-week period, the pig was largely denuded except for a small amount of hair around the head and neck and some over the back. The greatest area of alopecia over the back was down the midline. Figure 1 shows the hair loss exhibited by pig 95 at the end of the experiment. Pig 95 also exhibited a spasticity of the hind legs. By observation, it appeared that it was painful for the pig to stand on its hind legs. Figure 2 shows the cracking of the feet which occurred in pig 95. The feet were sore, rough, cracked and occasional bleeding was observed. The cracking of the feet was observed at about the same time the hair loss began and the feet became progressively worse during the remainder of the feeding period. Since the pigs were kept on a wire-mesh floor, this may have aggravated the condition of the cracked feet. However, the pigs receiving the same ration plus biotin showed no evidence of cracked feet. There seemed to be a correlation between the amount of hair loss and the severity of cracked feet. The syndrome exhibited by pig 95 was also shown by the other pig (105) fed the same ration in lot IIAA. The syndrome in pig 105 did not develop as rapidly or reach the severity of that in pig 95. Both pigs showed erratic appe-

tites which limited the feed consumption of the other pigs. Both pigs exhibited a hypersensitivity when forced to move.

The addition of biotin to the basal ration plus sulfathalidine (lot IIIBB) prevented the syndrome described for pig 95 fed the same ration minus biotin, and also increased growth by



Fig. 1 Left, pig 95 fed basal ration + sulfathalidine (lot IIAA). Right, pig 106 fed same ration + biotin (lot IIIBB). The syndrome of pig on left was prevented by biotin as is evidenced by normal appearing pig on right. Note spasticity of hind legs, hair loss, dermatosis of skin characterized by dryness, roughness, and a brownish exudate.



Fig. 2 Pig 95 (lot IIAA). Note cracked feet. The feet were sore and bled at times. This condition was prevented by biotin.

57% as is shown in table 3. The syndrome produced above by adding sulfathalidine to the ration and prevented by biotin is very similar to the syndrome produced by feeding desiccated egg white in the ration of the pig (Cunha et al., '46). The syndrome produced by Cunha et al. ('46) was prevented by

intramuscular injection of biotin. This might indicate that the effect of biotin in preventing the syndrome described above was direct and not by stimulating the intestinal synthesis of other factors.

Of much interest is the finding that the addition of inositol to the pigs fed the basal ration plus sulfathalidine in lot IIBB alleviated to a large extent the deficiency symptoms which were prevented by biotin in lot IIIBB. The inositol alleviated the degree of hair loss and the severity of cracked feet which were very marked when biotin was not included in the ration. This tends to show that inositol alleviated biotin deficiency symptoms. A possible explanation for this, is that inositol stimulated certain organisms in the intestinal tract to synthesize biotin.

When sulfathalidine was added to the basal ration, the magnitude of the daily gains were decreased in the following order by the supplements fed (table 3): (1) 1-20 liver powder plus biotin; (2) inositol; (3) 1-20 liver powder, and (4) biotin. Inositol stimulated growth more than biotin, although biotin was more effective in preventing the syndrome caused by adding sulfathalidine to the ration. The fact that the addition of 1-20 liver powder (lot IB—table 2) to the basal ration did not stimulate growth might indicate that the liver, at the level fed and under the conditions of this experiment, did not supply additional factors at an adequate level for stimulating growth. However, this does not mean that the pig does not need unknown factors. Work of McRoberts and Hogan ('44) has shown that the very young pig needs unidentified factors for growth. The work of Cunha, Ross, Phillips and Bohstedt ('44) indicated that an unidentified factor or factors is needed by the sow for reproduction and lactation.

Hegsted et al. ('40, '42) reported that a deficiency of biotin with the chick resulted in hemorrhagic cracks and incrustations in the bottom of the feet. In many cases, the toes became necrotic and sloughed off. Biotin prevented a cracking of the feet in the pig in the trial reported herein as well as in the experiment when desiccated egg white was fed in the ration

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COBALT METABOLISM STUDIES

II. PARTITION OF RADIOACTIVE COBALT BY A RUMEN FISTULA COW¹

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Little is known concerning the role of cobalt in animal nutrition; however, the contention that cobalt is essential in the ruminant diet is supported by the increasing numbers of field observations reporting widespread deficiency areas (Beeson, '45). The situation with regard to the essentiality of cobalt in the nutrition of non-ruminants is less clear. Although the extremely small requirement of the animal for cobalt has minimized the opportunities of obtaining the needed information with the ordinary methods of chemical analysis, the use of radioactive isotopes has provided a method by means of which physiological amounts of the element can be employed and traced. The literature on cobalt in animal nutrition has been comprehensively reviewed by Russell ('44).

Emphasis has been placed on experiments with cattle, first because it was logical to use a species for which cobalt is known to be essential and secondly, because of the economic importance of cobalt deficiency diseases in the livestock industry. This paper reports observations on the fate of labeled cobalt administered to a rumen fistula cow, and confirmatory data obtained from range cattle by the slaughter method.

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EXPERIMENTAL

The radioactive cobalt was supplied by the Massachusetts Institute of Technology-Radioactivity Center and was prepared by bombardment of iron with deuterons. It consisted of a mixture of three cobalt isotopes ranging from 65 to 270 days half life and contained 5.45 mg cobalt as cobalt chloride with an initial specific activity of 3303 μg radium gamma-ray equivalent per mg.

The jugular injection experiment was performed about 1 month after the preparation of the radioactive material, while the rumen administration part of the study was carried out after an interval of about 4 months; in the first case the measurements were sensitive to 0.0006 μg cobalt while in the latter work the sensitivity was 0.002 μg cobalt. No inert cobalt was used in preparing the doses. The detection procedure consisted essentially of ashing the sample, bringing the ash into solution and quantitatively electroplating the cobalt onto a copper disk which was used for the actual measurement with Geiger-Mueller counter apparatus. The details of this method have been described by Comar, Davis and Taylor ('46).

The rumen fistula cow, belonging to the Michigan State College herd, was a 1000 lb. Holstein in good health and general condition. It was born on August 28, 1941, and calved the last time on February 24, 1945. A 24-hour collection gave 11.0 kg urine and 22.9 kg feces. The rumen contents, removed 1 day at the usual sampling time, weighed 161 lbs. The blood volume was 27.6 l as determined with Vital red. Milk production during the injection experiment was about 18 lbs. a day; however, it had fallen to about 5 lbs. a day during the rumen administration study. The animal was on a ration that consisted of 15 lbs. alfalfa, 20 lbs. corn silage, 6 lbs. corn and 50 gm salt.

The size of samples used and the preservatives were as follows: 20-40 gm blood, potassium oxalate; 40-80 gm rumen contents, formaldehyde; 40-80 gm feces, concentrated HCl; 30-120 gm urine, toluene; about 300 gm milk, formaldehyde;

and 200-300 gm saliva, formaldehyde. As a rule the larger size samples were used in cases where there was little or no activity. The radioactive cobalt solutions were prepared in Gainesville and shipped to East Lansing for administration to the animal. The preserved samples were returned to Gainesville for the analyses.

On July 20, 1945, at 7:45 A.M. 10 ml of a solution containing 174 μ g labeled cobalt as cobalt chloride were injected into the jugular vein of the animal. A blood sample from the opposite side was taken 5 minutes later and the samples then collected periodically, before feeding whenever possible.

On October 11, 1945, at 7:45 A.M. 2 ml of a solution containing 174 μ g labeled cobalt as cobalt chloride were introduced, by capsule, directly into the rumen and the samples collected periodically. Since the behavior of cobalt in the rumen was of most interest, it was felt that direct rumen administration was preferable to avoid any possibility of the capsule by-passing the rumen, as might occur after oral administration.

RESULTS AND DISCUSSION

Table 1 presents the data on the fate of the administered labeled cobalt. In each case a complete set of samples was taken before the dosage, none of which showed any activity. This indicated, as expected, that no naturally occurring or extraneous radioactive materials were involved, but of more importance, that there was no residual activity at the time of the rumen administration due to the previous injection.

It is interesting to note the rapid disappearance of the injected cobalt from the blood stream; after 5 minutes only about 6% of the dose was present in the blood of the animal. There appeared to be a certain periodicity, with the blood value falling to 0 at 54, 175 and 319 hours after injection. When the cobalt was administered into the rumen, none was detectable in the blood.

None of the injected cobalt was found in the rumen contents. When the cobalt was introduced directly into the rumen about 82% of the dose was found in the rumen con-

TABLE 1

Partition of radioactive cobalt by rumen fistula cow (dosage 174 μ g cobalt).

SAMPLE	JUGULAR INJECTION			RUMEN ADMINISTRATION			SAMPLE	JUGULAR INJECTION			RUMEN ADMINISTRATION		
	Hours after dosage	μ g Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample ¹	Hours after dosage	μ g Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample ¹		Hours after dosage	μ g Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample ¹	Hours after dosage	μ g Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample ¹
Blood	1 ¹ / ₂	0.034	5.6	9	"	0	Feces (con.)	197	0.0010	0.3	216	"	0
	1	0.048	7.9	24	"	0		217	0.0017	0.1	240	"	0
	8	0.024	4.0	48	"	0		247	0.0016	0.3	264	"	0
	32	0.0037	0.6	72	"	0		271	0.0012	0.2	288	"	0
	54	"	0.0	96	"	0		295	"	0.0	312	"	0
	80	0.018	3.0	120	"	0		319	0.0013	0.1	336	"	0
	104	0.018	3.0	144	"	0		344	0.0012	0.2			
	128	0.0053	0.9	168	"	0	Urine	11	2.4	7.9	37	"	0
	152	0.0068	1.1	192	"	0		71	0.99	27.8	87	"	0
	175	"	0.0	216	"	0		32	0.080	34.2	24	"	0
	197	0.014	2.3	240	"	0		54	0.025	3.1	48	"	0
	217	0.0074	1.2	264	"	0		80	0.073	3.4	72	"	0
	247	0.0094	1.6	288	"	0		104	0.064	4.4	96	"	0
	271	0.0056	0.9	312	"	0		128	0.012	2.4	120	"	0
	295	0.0035	0.6	336	"	0		152	0.017	0.9	144	"	0
	319	"	0.0					175	0.0058	0.7	168	"	0
	344	0.0080	1.3					197	"	0.0	192	"	0
Rumen contents	14	"	0	37	0.19	81.6		217	0.022	0.6	216	"	0
	8	"	0	9	0.13	56.3		247	0.0054	1.1	240	"	0
	32	"	0	24	0.12	51.2		271	0.011	0.5	264	"	0
	54	"	0	48	0.049	20.7		295	0.0089	0.6	288	0.0067	0.5
	80	"	0	72	0.022	9.4		319	0.018	0.9	312	"	0
	104	"	0	96	0.0096	4.0		344	0.010	0.9	336	"	0
	128	"	0	120	0.0043	1.8	Milk	87	0.0007	0.03	9	"	
	152	"	0	144	0.0030	1.3		81	0.0070	0.3	21	"	
	175	"	0	168	0.0010	0.4		249	0.0025	0.1	57	"	
	197	"	0	192	"	0					105	"	
	217	"	0	216	"	0					249	"	
	247	"	0	240	"	0					369	"	
	271	"	0	264	"	0	Saliva	1	0.0006		9	"	
	295	"	0	288	"	0		247	"		101	"	
	319	"	0	312	"	0					264	"	
	344	"	0	336	"	0					344	"	
Feces	14	"	0	87	0.014	0.3							
	8	0.020	0.4	24	0.42	18.2							
	32	0.0089	2.0	48	0.17	39.5							
	54	0.0057	0.9	72	0.024	12.8							
	80	0.0051	0.8	96	0.021	2.9							
	104	0.0064	0.8	120	0.010	2.1							
Feces	128	0.0018	0.5	144	0.010	1.3							
	152	0.0037	0.4	168	0.003	0.8							
	175	0.0035	0.5	192	"	0							

¹ Estimated percentage of dose: in total blood volume of 27.6 l; in total rumen contents of 161 lbs.; based on daily feces excretion of 22.9 kg; based on daily urine excretion of 11.0 kg; based on daily milk production of 18 lbs.

" Amount in sample less than 0.0006 μ g.

" Amount in sample less than 0.002 μ g.

tents after 3½ hours. The rate of disappearance from the rumen is shown, and after 168 hours less than 0.5% of the original dose was present in the rumen contents. These percentage values are based on the single determination of rumen contents at 161 lbs., however, small uncertainties would not change the picture.

Since it was not possible to make 24-hour collections throughout the experiments, the calculated values for the per cent of dose in the total daily excretions must be considered as estimates only. However, they do indicate the order of magnitude and are helpful in the interpretation of the data. Only about 7% of the injected cobalt appeared in the feces; other data have indicated the probability that much of the injected cobalt reached the intestinal tract via the bile. Where the cobalt was introduced into the rumen, large amounts appeared in the feces within 48 hours, with about 65% of the dose being accounted for. The disappearance of the cobalt from the rumen contents coincided with its appearance in the feces, which may be assumed to indicate lack of absorption.

Large amounts of the injected cobalt were rapidly eliminated in the urine. At 1½ hours after injection the concentration in the urine was very high, and after 32 hours about 65% of the dose had been eliminated by this path. In the measurements on the urine after rumen administration the sensitivity was such that no activity was found even though samples of about 100 gm were used; however, 311 gm of a mixture of the first five samples gave a value of 0.0006 µg per 100 gm fresh weight, which indicated that an extremely small but definite amount of the cobalt was present in the urine.

Small amounts of the injected cobalt were found in the milk. There were not enough milk samples to give a reliable indication as to the proportion of the dose eliminated by this path; however, it would seem that not more than a few per cent, if that much, could be accounted for in the milk. No activity was detectable in the milk samples after the rumen administration; it should be pointed out that although the

sensitivity was lower in these determinations, amounts comparable with those found in the injection milk samples would have been detected.

An extremely small amount of cobalt was found in the saliva 1 hour after injection. It is not surprising that the cobalt carried by the saliva after injection did not show up in the rumen contents when it is considered that the amount in the saliva was small to start with, and that the rumen contents were considerably diluted with food and water. No cobalt was found in the saliva after the rumen administration.

These observations with the rumen fistula cow have been confirmed by data from other experiments in which the animals were sacrificed at varying time intervals after the administration of the labeled cobalt. The values reported in table 2 were obtained with the following range cattle: cow no. 6, about 5 years old, weight 300 lbs.; steer no. 2, about 2 years old, weight 230 lbs.; heifer no. 3, about 1½ years old, weight 195 lbs.; cow no. 7, about 8 years old, weight 525 lbs., and steer no. 1, about 2 years old, weight 250 lbs.

TABLE 2

Partition of radioactive cobalt by range cattle (slaughter method).

ANIMAL	DOSE μG CO	MODE OF ADMINIS- TRATION	TIME AFTER ADMINIS- TRATION	PER CENT OF DOSE IN WHOLE SAMPLE		μG COBALT PER 100 GM FRESH WEIGHT	
				Blood	Rumen contents	Large in- testine contents	Urine (not ex- creted)
<i>hours</i>							
Cow no. 6	260	Injection	2	3.8	0.17	0.0064	2.2
Steer no. 2	1330	Injection	24	3.0	0	0.40	5.3
Heifer no. 3	2400	Injection	264	0.44	0	0.12	1.2
Cow no. 7	218	Oral	14½	0	63.8	0.90	0.0015
Steer no. 1	1330	Oral	240	0	0	0	

These animals, in poor condition, were brought to Gainesville from an area in Florida considered deficient in some of the essential minerals. They were then fed on a ration consisting of redtop hay harvested from a low cobalt area, corn grown on a low cobalt area, a protein supplement of dried skim milk, and a phosphorus supplement of tri-calcium phos-

phate. Analyses showed that this feed contained less than 0.01 p.p.m. cobalt which is considered below the maintenance level. For about 6 months under these conditions most of these animals remained in poor condition and gave an appearance of emaciation, rough hair coat, and extreme weakness.

Although these range animals were presumably in a condition of cobalt deficiency the results obtained were in good overall agreement with those reported for the rumen fistula cow. About 3 to 4% of the injected cobalt was present in the blood after 24 hours with the value falling to less than 1% after 264 hours. None of the orally administered cobalt was found in the blood; in the case of cow no. 7, 226 gm of blood were analyzed and the measurements were sensitive to 0.0015 μ g cobalt. Here again, with the exception of cow no. 6, none of the injected cobalt was detected in the rumen contents. The cobalt in the rumen contents of cow no. 6 probably reached there via the saliva since this animal received no food or water after the injection and its rumen contents weighed only 26 lbs. About 64% of the orally administered dose was present in the rumen contents of cow no. 7 sacrificed after 14½ hours, while none was found after the 240-hour interval (steer no. 1). The values for large intestine contents and urine (not excreted) agree well with the corresponding values for the rumen fistula cow, especially when it is considered that the range animals were consuming less feed and water, and that steer no. 2, heifer no. 3 and steer no. 1 received larger doses.

It is apparent that the cobalt requirement of the ruminant must be small indeed if retention by the animal is any criterion. Comar, Davis and Taylor ('46) have reported that 10 days after the injection and oral administration of 1 to 2 mg cobalt less than 5 and 1%, respectively, were retained by the cattle. McCance and Widdowson ('44) suggest that the element probably acts upon some of the organisms in the rumen and not on the host, a contention which is supported by observations that cobalt was not curative when given to sheep by injection, and that horses do not apparently suffer from

cobalt deficiency diseases. The findings, reported here, that no significant amounts of injected cobalt reach the rumen contents and no significant amounts of rumen ingested cobalt reach the blood, fit into this picture. The data indicate that cobalt supplements should be supplied at least every week if it is desired to maintain the cobalt level in the rumen contents of animals on feed deficient in this element.

Other studies with radioactive cobalt, many of which are in progress, will be required to permit a decision as to whether cobalt is essential for the normal functioning of haematopoietic or other organs.

SUMMARY

1. When $174\ \mu\text{g}$ labeled cobalt were injected into the jugular vein of a rumen fistula cow about 6% of the dose was present in the blood after several hours with the value falling to 1% or less after 10–15 days. None was found in the rumen contents, about 7% appeared in the feces, and about 65% was found in the urine with large amounts being rapidly eliminated by this path. Very small amounts were found in the milk and saliva.

2. When $174\ \mu\text{g}$ labeled cobalt were introduced directly into the rumen none was detected in the blood. About 82% of the dose was present in the rumen contents after 4 hours with the amount decreasing regularly to less than 1% after 7 days. Over 65% was accounted for in the feces and only extremely small amounts in the urine. No cobalt was detected in the milk or saliva.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

IX. THE DIGESTIBILITY OF MARGARINE FAT IN HUMAN SUBJECTS¹

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The first essential for a food to be considered of high nutritive value is that it be relatively completely digested and absorbed. In an extensive series of experiments carried out by the Office of Home Economics of the United States Department of Agriculture on a wide variety of animal and vegetable fats, it was found that most of the seventy odd fats studied were digested to the extent of 95% or better by human subjects (Langworthy, '23). The exceptions to the almost complete digestibility were fats which had melting points of 50°C. or above such as mutton fat (Langworthy and Holmes, '15), oleostearin (Holmes, '19), deer fat (Deuel and Holmes, '22) and some almost completely hydrogenated fats melting from 52 to 60°C. (Deuel and Holmes, '21).

There is practically no experimental evidence on the digestibility of the modern margarines, most of which are composed of such hydrogenated fats as cottonseed, peanut or soybean oil. Holmes ('25) found that three different types of margarine commercially available in 1915 which were prepared from animal fats were highly digestible. This was true for the sample which contained a considerable portion

¹ This work was carried out under a research grant from The Best Foods, Inc.

of oleostearin even though this fat when fed as the sole fat in the diet, had been shown to be digested to the extent of only 80%.

Since practically complete digestibility has previously been reported for partially hydrogenated cottonseed, corn, and peanut oil with melting points under 50°C. (Deuel and Holmes, '21) as well as a comparable series of blended hydrogenated fats (Deuel and Holmes, '22) (prepared from completely hydrogenated oils mixed with sufficient of the untreated oil to bring the mixture to the desired melting point), there is no reason to suppose that the modern margarines would behave differently. The present tests were carried out in 1938 to determine whether such a supposition is correct. Because of the increasing importance of margarine in the present day diet, these results are now being reported.

EXPERIMENTAL

The digestibility experiments were performed using procedures identical with those employed in the studies of the Department of Agriculture referred to earlier. The subjects were students and technicians closely associated with the Department of Biochemistry who were known to be reliable. There were 5 men and 5 women subjects. All were in normal health.

The experiments were carried out over a 3-day period. During this period the subjects ate a blanc mange pudding consisting of skim milk, cornstarch, sugar, salt, caramel and vanilla into which margarine fat or butter fat was incorporated. In addition to the pudding, whole wheat biscuits, oranges and black coffee with sugar were used. Seven tests were made with margarine fat and three with butter fat. The amount of each food eaten was determined and the total intake of the various foodstuffs calculated from these data. The stools from the experimental period were separated by markers taken with the first meal of the experimental diet and with the first meal following the conclusion of the test.

The stools were dried to constant weight at 95°C. and analyses were made on the pulverized samples for lipid, protein and ash. Carbohydrate was estimated by difference. In estimating the digestibility of the fat, a correction for metabolic fat in the feces was made by multiplying the weight of dry feces by 9.89 (Langworthy and Holmes, '15). The margarine fat was separated from a commercial margarine which was identified as being made from domestic vegetable oils and as having a melting point well below body temperature (94-95°F. Wiley). This melting point is about the average of the margarines currently available, practically all of which vary between 91°F. and 98°F. (Wiley). The butter fat was prepared from a butter widely used in this area.

RESULTS

The results of the tests are summarized in table 1.

TABLE 1

Summary table of digestibility of margarine fat and butter fat in normal men and women.

SUBJECT	SEX	WEIGHT	FAT EATEN	DIGESTIBILITY OF THE DIET AS A WHOLE				CORRECTED COEFFICIENT OF DIGESTI- BILITY OF FAT
				Protein	Fat	Carbohydrate	Ash	
Experiments with margarine fat								
		lbs	gm	%	%	%	%	
A.B.	M	185	331.8	82.2	94.9	94.8	64.7	98
H.B.	M	157	218.6	75.0	90.3	96.5	56.2	94
W.B.	F	122	161.2	56.4	91.3	95.8	50.7	96
B.B.	F	121	332.0	78.8	94.2	96.9	70.9	96
L.H.	F	122	215.5	83.1	94.4	95.2	65.0	97
V.H.	M	132	292.5	92.5	97.1	97.9	81.4	99
S.M.	F	112	264.3	83.9	93.1	96.6	72.7	97
Average			259.5	78.8	93.6	96.3	65.9	97
Experiments with butter fat								
M.G.	F	104	191.0	71.8	96.4	92.7	60.8	100
J.H.	M	136	80.0	50.5	90.1	95.2	30.2	99
L.K.	M	152	167.3	74.1	88.5	97.5	57.7	92
Average			146.1	65.5	91.7	95.1	49.6	97

The average coefficient of digestibility of the seven experiments on margarine is 97 which is identical with the average obtained in the three tests on butter. The average intake of margarine fat was 260 gm for the experimental period with a maximum of 111 gm per day. The average intake of butter was 146 gm for the 3-day period which was somewhat lower than the fat intake in the margarine experiments. The results of the butter tests agree with those reported earlier (97.0) by Langworthy and Holmes ('15). There was no evidence of alteration in the absorption of other components of the diet with either diet. The subjects remained in normal health for the duration of the tests. These data indicate that from the standpoint of digestibility, margarine can be considered as containing a fat of high nutritive value.

SUMMARY

Margarine fat composed of hydrogenated domestic vegetable oils was found to be digested to an average of 97% by normal men and women which was an identical value obtained in three tests on butter fat. There was no evidence of any unpleasant physiological effects when a maximum of 111 gm of margarine fat was ingested daily.

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THE NICOTINIC ACID, BIOTIN, AND PANTOTHENIC ACID CONTENT OF COWS' MILK¹

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THREE FIGURES

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Milk has long been regarded as one of our most efficient foods, particularly in the case of infants and children where the demands for growth and development are at a peak. While human milk might be considered as the normal and preferred food for the human infant, many babies cannot be, or are not, breast fed. In any case, cow's milk sooner or later becomes one of the most important constituents of the diet of infants and children.

Studies at the Research Laboratory of the Children's Fund of Michigan (Maey et al., '45) are providing much information on the nutrient composition of breast milk under various known or controlled conditions. Some of the corresponding data for cow's milk are already available in the scientific literature. The present study has been planned to add to those data, and designed to parallel the work of the Detroit group with respect to some of the causes of variation in the nutrient composition.

The factors in human milk studied by the Detroit group have included vitamin A and carotene, ascorbic acid, thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotin. With respect to cow's milk, there is considerable information on vitamin A and carotene (Shaw et al., '37; Kramer et al., '38; U. S. Department of Agriculture, '45), ascorbic acid (Kon

¹ This study was aided by a grant from the Nutrition Foundation, Inc.

and Watson, '37; Whitnah and Riddell, '37), thiamine (Houston, Kon and Thompson, '40), and riboflavin (Theophilus and Stamberg, '45; Whitnah, Kumerth and Kramer, '38; Johnson, Maynard and Loosli, '41). Since completion of this work, Pearson and Darnell ('46) have published the results of a study of the thiamine, riboflavin, nicotinic acid, and pantothenic acid in cow's colostrum and milk. Except for isolated assay values, there is no other information available on the nicotinic acid, pantothenic acid, and biotin content of cow's milk. We have attempted to provide such data by a systematic study of the causes of variation of these vitamins in cow's milk.

METHODS OF ASSAY

The assay methods used were basically the same as those published elsewhere, though somewhat modified in details; first, in order to be able to use the same culture media and stock solutions in assays for all three vitamins; and second, to take advantage of apparent improvements suggested in more recent papers.

Our methods were designed for assay of only one type of sample; namely, milk. They are not necessarily applicable to other samples.

Recovery experiments run by adding known amounts of a vitamin to a previously assayed milk sample gave satisfactory results for all three vitamins.

Cultures and inoculum

The organism for assays for all three vitamins was *Lactobacillus arabinosus* 17-5. The cultures were transferred in the manner described by Krehl, Strong and Elvehjem ('43), but the compositions of the media were somewhat different. It was felt that they were kept in a more vigorous condition by carrying them in an agar medium containing 1% Difco tryptone, 0.1% glucose, 0.2% phosphate, 0.3% calcium carbonate, and an amount of liver extract representing 0.05 pound of liver per liter of medium. For subculturing the organism from one stab culture to another, and in growing inocula, the

broth medium used contained 1% Difco yeast extract, 0.1% glucose, and 0.5% phosphate.

The latter medium could be used in growing inocula in assays for all three vitamins, but its use required washing of the cells. The cells of a 24-hour culture were centrifuged down, resuspended in 10 ml of sterile 0.9% saline solution, and again centrifuged and resuspended. In the nicotinic acid assays, one drop of the latter saline suspension was used to inoculate each tube. In the biotin and pantothenic acid assays, it was diluted ten-fold before use. In these assays, it was found to be essential that the final inoculum be homogeneous. Any large clumps of cells were allowed to settle or were removed by slow centrifugation before diluting an aliquot of the supernatant suspension.

Basal media

The detailed composition of the media used for the assays is given in table 1.

The hydrolyzed casein was prepared by the method of Krehl, Strong and Elvehjem ('43). The inorganic salts solutions A and B were the same as those of Snell and Wright ('44). The alkali-treated peptone was prepared as described by Strong, Feeney and Earle ('41). The yeast extract was a 10% solution in 0.5 N sodium hydroxide, autoclaved for 30 minutes, neutralized and filtered. We found it necessary to avoid using certain samples of Difco yeast extract which were not freed of pantothenic acid activity by this treatment. This was apparently due to the presence of the alkali-stable pantothenic acid-active material reported by Neal and Strong ('43). The rice polish concentrate was a 5% solution (based on the starting material), prepared by suspending the material at pH 1.3 to 1.5, filtering it, and putting the filtrate through a column of charcoal, and neutralizing.

Procedure

The assay tubes were set up in the usual way with graded levels of standard for the standard curve and various levels

of samples. A special syringe with a small bore tip was used to add the 5 ml of basal medium to the 5 ml volume of standard or sample and water. It was thus possible to add it with sufficient force to thoroughly mix the contents of the tube. Glass caps were used on the assay tubes in place of the usual cotton plugs.

TABLE 1
Compositions of the basal media.

COMPONENT	MEDIA FOR		COMPONENT	MEDIA FOR	
	Nicotinic acid and biotin	Pantothenic acid		Nicotinic acid and biotin	Pantothenic acid
Hydrolyzed casein	10 gm	4 gm	Nicotinic acid	2 mg ¹	
Alkali-treated peptone		10 gm	Thiamine	800 µg	
Alkali-treated yeast extract		2 gm	Pyridoxine	1 mg	
Charcoal-treated rice polish concentrate		20 ml	Calcium pantothenate	1 mg	
l-Cystine	400 mg	200 mg	Riboflavin	2 mg	
l-Tryptophane	200 mg	200 mg	Biotin	3 µg ²	
l-Asparagine hydrate	500 mg	500 mg	p-Amino benzoic acid	500 µg	
Adenine	20 mg	20 mg	Glucose	40 gm	40 gm
Guanine	20 mg	20 mg	Sodium acetate	40 gm	28 gm
Uracil	20 mg	20 mg	Inorganic salts A	10 ml	10 ml
			Inorganic salts B	10 ml	10 ml
			Distilled water to make 1 liter ³		

¹ Omitted in nicotinic acid assays.

² Omitted in biotin assays.

³ The final medium has half this strength, since an equal volume of sample (or standard) plus water is present.

The titration was carried out electrometrically, using a quinhydrone electrode. By using a Ag-AgCl reference electrode in normal KCl, the potential and galvanometer reading fall to zero at a pH of about 7.8. The sharpest end point was found to be obtained at this pH, using the basal medium described previously.

Preparation of samples

Soon after being drawn, the milk samples were refrigerated, and within 24 hours (in nearly all cases) they were frozen in 5 ml portions for storage at -13 to -16°C . A small amount of each sample was reserved unfrozen for analysis for fat and total solids by the Mojonnier methods. The stored samples were seldom held for more than a month before being assayed, and experiments showed that the concentrations of nicotinic acid, biotin, and pantothenic acid did not decrease in a much longer period than this.

Assay results with these three vitamins were not increased by treatment with acid or enzymes (polidase, mylase, clarase, or takadiastase plus papain), so that there is apparently no bound nicotinic acid, biotin, or pantothenic acid in milk. Since agreement of results at different levels of the samples was quite satisfactory without any pretreatment other than melting them and diluting them to the proper extent, no other pretreatment was used.

DESCRIPTION OF SAMPLES

In order to isolate the effects of both stage of lactation and season on the vitamin level of the milk, samples were obtained at various stages of lactation from cows freshening at different seasons of the year. For each cow, samples were taken twice daily for the first 2 or 3 days after freshening; on the next 2 days, composite samples were made from the morning and evening milkings; following these, such composite samples were taken at gradually increasing intervals, so that after the first month composites were obtained once a month. When the cow began to dry off, samples were taken more frequently. The total milk yield represented by each sample was recorded. Per cent fat and total solids, as well as the three vitamins, were determined for each sample.

Three cows (nos. 60, 38, and 46) were used from November 3, 1943, when they were approaching the end of their lactation periods, till they dried off near the beginning of the following year. Milk samples from another group of four cows (nos. 16,

68, 7, and 33) were analyzed from the time they freshened in November and early December, 1943, throughout their lactation periods until they dried off in August and September, 1944.² The cows of the first group and one other (no. 15) were studied from the time they freshened in March and early April, 1944, till the experiment was terminated at the end of the year, or until an earlier end of lactation was necessary.³ Two others (Venus and Delle) were followed from the time of freshening at about September 1, 1944, till the last of January, 1945. Cow no. 33 from the second group, freshened again on November 17, 1944, and samples of her milk were analyzed till the end of the year. All of these cows were Holsteins.

Because of difficulties encountered in obtaining satisfactory pantothenic acid assays during the early part of the studies, many of the earlier samples either were not assayed for this vitamin, or the assay results were excluded from consideration.

Since variations in the feed received by the cows appeared to have little effect, in most cases, on the vitamin content of the milk, it seems unnecessary to describe the feeding regime in detail. Cows 16, 68, 33, 46, and 7 were on feeding experiments unconnected with this study during much of their respective lactation periods. Their feeds were varied frequently with respect to hay, corn silage, and the grain mixture. The changes involved particularly the fat content of the rations. All the other cows were fed standard rations according to normal practices of good herd management. The most clear-cut and sustained change undergone by all these cows was their being put on and taken off pasture.

² Cow no. 7, however, was still being milked when the experiment was terminated at the end of the year.

³ Cow no. 60 had a foot infection throughout this lactation period. This kept the milk yield low, and she was finally killed in August, 1944.

Cow no. 38 became sick in September, 1944, and was dried off without further samples being taken.

Cow no. 15 developed mastitis in November, 1944, and was killed. (The proportion of mortalities encountered in this study is not unusual in dairy herds.)

RESULTS

The accuracy usually claimed for microbiological assay methods allows for errors of up to 10%. While the nicotinic acid assay method, as used in this study, was probably much more accurate than this in nearly all cases, the figure of 10% gives a reasonably conservative basis from which to draw conclusions about the levels of each of the vitamins.

Variation between cows

There is considerable variation in the vitamin content of the milk depending on the characteristics of each individual cow. This variability is particularly great in the case of biotin. The average biotin levels of all milk samples during the first 4 months after the colostrum period for each of eight cows were 11, 12, 15, 17, 18, 20, 28, and 37 μg per liter. The disparity among individual cows in this respect was much greater during the colostrum period. These facts are also made evident by the relative large values of standard deviations of the means shown in figure 2, in which the relation of biotin level to stage of lactation is represented. Of all milk samples assayed, the maximum level for biotin was 56 μg per liter and the minimum was 2 μg per liter. The range for colostrum samples was from 0.6 to 91 μg per liter. The figures given by Hodson ('45) also show a very wide range. The variability of pantothenic acid levels of the milks of individual cows was considerable, but not so great as for biotin. The average calcium pantothenate levels for the 4 months following the colostrum period for each of six cows were 2.7, 2.7, 3.0, 3.8, 4.5, and 4.6 μg per ml. Similar averages for nicotinic acid levels from each of ten cows were 0.42, 0.48, 0.54, 0.62, 0.69, 0.71, 0.80, 0.85, 0.96, and 1.02 μg per ml. Attempts to relate these variations to milk yield, age of the cow, caloric value of the milk (calculated from its fat and solids content), leucocyte count of the milk, or the per cent of fat or other solids were unsuccessful.

Day-to-day variations

There were found to be day-to-day variations in the vitamin levels of the milk from any given cow. This was not particularly marked in the case of nicotinic acid. Daily composite milk samples were obtained on 4 consecutive days from one cow and on 9 consecutive days from another, and an average difference in the nicotinic acid level from 1 day to the next of 7% was found for each series. The pantothenic acid variations for the same series of milk samples were larger, being 10%. With biotin, the changes were quite unpredictable. The first 4-day series gave biotin levels of 21, 23, 21, and 22 μg per liter, but the cows whose milk was assayed on 9 consecutive days gave values of 30, 40, 21, 17, 22, 21, 21, 30, and 29 μg per liter on those days. We are aware of no reason for either constancy or change in this respect. This inconsistency was unexpected, and was not considered in planning the experiment. It obviously decreases the value of composite samples from a single day's milkings when taken relatively infrequently.

Seasonal variation

The relation of vitamin level of the milk to the time of year is shown in figure 1. These curves show the average levels in the milks of all cows being studied at the time for which a point is given. Such averages were determined at 20-day intervals. The level on any particular date for a given cow was obtained from the individual curve for that cow, drawn by connecting points representing the samples taken. Colostrum samples and samples taken at the time of drying off were not included in the averages. The number of cows represented by any one point varied, since certain cows were freshening and others were not being milked at various times throughout the study. This factor accounts for some of the irregularities in the curve. For example, the point for March 30 on the nicotinic acid curve was relatively high since between then and the preceding point, a cow with a relatively low level died, while another with a relatively high level freshened. The

trend of the nicotinic acid curve is clear, and is believed to be of real significance. Throughout late winter and early spring the nicotinic acid content of the milk gradually decreased until May. From then on, a rise set in until October. On May 9, the range of values for eight cows was 0.22 to 0.77 μg per ml, and on October 16, the range for five cows was 0.56 to 0.96 μg per ml. These points roughly correspond with the time at which

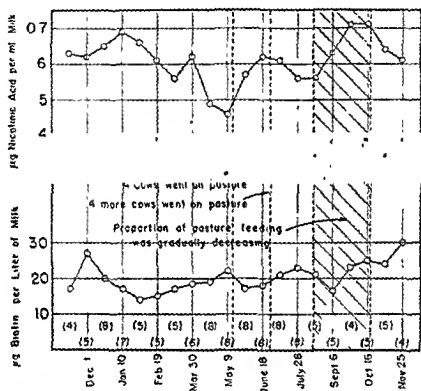


Fig. 1 Seasonal variation of the mean biotin and nicotinic acid levels of cows' milk. The figures in parentheses give the number of cows represented by each point.

the cows first went on pasture and the time of complete cessation of pasture feeding. The irregularities of the biotin curve have no apparent cause. When one considers the variability between cows, the fact that different cows are included in the averages giving the several points, and the day-to-day variation possible for a cow, the irregularities are probably of little significance. The pantothenic acid study was not continued over a long enough period, and differences in stage of lactation at any one date were not sufficient to make any complete analysis of the seasonal variation in pantothenic acid.

Variation with stage of lactation

The relation of the milk, nicotinic acid, and biotin levels to the stage of lactation is shown in figure 2. These curves give the average levels at various intervals throughout the lactation period. They were obtained in the same way as the curves for seasonal variation except that, instead of determining the

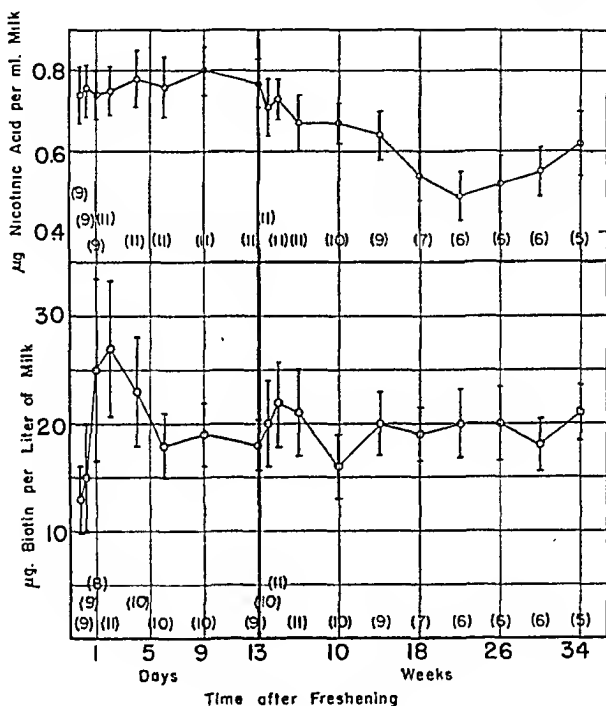


Fig. 2 Variation of mean nicotinic acid and biotin levels of cows' milk with stage of lactation. The standard deviation of the mean is indicated by the spread above and below each point. The figures in parentheses give the number of cows represented by each point.

mean of the levels at corresponding dates from the curves for individual cows, the mean at corresponding points in the lactation period was determined. Again the number of cows represented by the points varies but the variation is much more regular. The number of samples represented by each point becomes smaller as the lactation period progresses because of

the early loss or drying off of some of the cows and the fact that others were not followed through the complete cycle. The standard deviation of the mean has been determined in each case and the values are shown with the curve. They give an indication of the significance of the curve. There appears to be a gradual fall in the nicotinic acid content of the milk throughout the lactation period at least up until the twenty-second week. The extreme variability of the biotin level during the colostrum period is apparent. The initial rise of biotin and subsequent fall to the normal level was quite characteristic

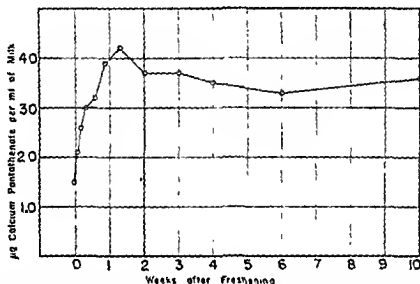


Fig. 3 Variation in mean pantothenic acid level of cows' milk with stage of lactation. Each point up to and including the third week represents seven cows, and the three remaining points represent six cows.

of the curves for the individual cows but the size of the standard deviations of the means makes the evidence for any relation with stage of lactation insufficient. The number of cows represented by the pantothenate curve (fig. 3) was too small to make a calculation of standard deviations worthwhile but the relation here was apparent. The general form of the curve was characteristic of each of the curves for individual cows except for the rise in the level at the end. From an inspection of the individual curves, it appears possible that the latter may be an effect of feed or season. Four cows which went on pasture at approximately this point in their lactation

period showed this secondary rise, while two others which freshened near the first of September did not show such an increase in pantothenic acid at this stage. The first part of the curve agrees very closely with the data of Pearson and Darnell ('46).

Variation with breed of cow

On three occasions a composite sample for each of four breeds was made at milking time using samples from representatives in the University herd. All breeds were therefore being treated alike. The cows involved on each occasion were largely the same individuals. The data appear in table 2.

TABLE 2
Breed comparison of milk vitamin levels.

DATE	BREED	NUMBER OF COWS REPRESENTED IN COMPOSITE	NICOTINIC ACID CONTENT	BIOTIN CONTENT	CALCIUM PANTO- THENATE CONTENT
			$\mu\text{g/ml}$	$\mu\text{g/l}$	$\mu\text{g/ml}$
2/16/45	Guernsey	8	0.64	19	4.2
3/ 1/45	Guernsey	12	0.50	22	2.2
3/16/45	Guernsey	12	0.65	18	2.6
2/16/45	Jersey	4	0.81	26	5.5
3/ 1/45	Jersey	3	0.76	22	3.3
3/16/45	Jersey	3	0.81	22	3.7
2/16/45	Brown Swiss	9	0.78	29	4.9
3/ 1/45	Brown Swiss	8	0.69	27	2.5
3/16/45	Brown Swiss	7	0.79	24	3.8
3/ 1/45	Holstein	16	0.81	16	2.3
3/16/45	Holstein	14	0.86	14	2.4

We do not consider the data sufficient to establish any significant differences in nicotinic acid content among the Jersey, Brown Swiss, and Holstein milks, but the Guernsey milk appears to be significantly lower. For both biotin and pantothenic acid the order of decreasing vitamin level was Brown Swiss, Jersey, Guernsey, and Holstein. The significance of this order is questionable, however, and the individuality of the cows may well have been more important than their breed.

Relation to other factors

We could not correlate the content of these three vitamins in the milk with other milk constituents determined or calculable, or with other factors. Relations considered were those with fat, total solids, milk-solids-not-fat, approximate caloric value, milk yield, and age of cow. However, there did appear to be some correlation between the pantothenic acid and biotin contents, though the nicotinic acid level seemed unrelated to the levels of the other two vitamins studied. When all milk samples, both normal and abnormal, were considered, the correlation coefficient between biotin and pantothenic acid levels was found to be 0.29. Statistical calculations indicate that the chances are less than one in a hundred that this correlation is fortuitous. Among the six cows mentioned previously in discussing the variation in pantothenic acid level among individual cows, the two highest in pantothenic acid were also the two highest in biotin, and the one lowest in biotin was one of the two lowest in pantothenic acid. If all points ⁴ including the colostrum periods, but excluding the drying off periods, are considered, the seven cows participating in the pantothenic acid study arrange themselves in exactly the same order with respect to each of the two vitamins. The curves showing the variation of mean vitamin level of milk with stage of lactation may be seen to be of a similar shape for both vitamins. That is, both start from an initially low level of vitamin in the milk, rise to a maximum level (somewhat earlier for biotin), and then fall to a normal level. The observation may be made (for whatever it is worth) that the four breeds studied have the same order, relative to the pantothenic acid and biotin contents of their milks. In none of these respects is nicotinic acid similar to the other two vitamins.

⁴ The points were obtained from the graphs for the individual cows at corresponding stages of lactation and mostly at times between those when actual samples were taken. Figure 2 was obtained by averaging such points.

Effect of pasteurization and other treatments

Nicotinic acid, biotin, and pantothenic acid in milk are apparently quite stable. As can be seen from table 3, no losses were found during pasteurization either by the batch, holder process, or by the continuous short-time, high-temperature process which included a deaeration step as developed at Cornell (Sharp, Guthrie and Hand, '40). There were no losses when a sample of pasteurized milk was exposed for 2 hours in a half-pint bottle to February noonday sunlight when

TABLE 3
Stability of vitamins in cow's milk.

	TREATMENT					
	Batch, holder pasteurization		Continuous short-time, high-temperature pasteurization			2 hours' exposure to sunlight
	1	2	1	2	3	
Nicotinic acid, μg per ml						
Before treatment	0.65	0.61	0.76	0.80	0.67	0.75
After treatment	0.64	0.64	0.80	0.82	0.70	0.76
Biotin, μg per liter						
Before treatment	22	21	25	24		24
After treatment	24	20	25	23		24
Calcium pantothenate, μg per ml						
Before treatment	2.3	3.6	2.6	3.5	3.4	4.2
After treatment	2.1	3.6	2.8	3.9	3.6	4.0

the temperature in the sun was 9°C. The nicotinic acid content did not decrease during at least 21 weeks' storage in the frozen state at about -14°C., and biotin was stable for at least 19 weeks under these conditions. No nicotinic acid was lost during 24 hours' incubation of milk at 37°C. under toluene (when diluted ten-fold with water).

The beneficial effect of milk in the pellagra-preventative diets used by early workers (Goldberger and Tanner, '24) was probably due, in part at least, to its aid in alleviating coexisting deficiencies of vitamins other than nicotinic acid, since the

nicotinic acid content of milk appears to be relatively small. However, it seemed worthwhile to investigate the possibility of the presence in milk of substances similar to those found in some materials by Krehl and Strong ('44) which become active with respect to nicotinic acid assay on treatment with alkali or strong acid. No increase in the assay value of a milk sample was found when it was incubated for 3 hours at pH 10 at 37°C.

DISCUSSION

The values found for the nicotinic acid content of milk in this study were lower than those reported by most other workers. From the literature, it would seem that a typical value would be in the neighborhood of 0.8 to 0.9 μg per ml, while the nicotinic acid level of milk samples in this study averaged about 0.61 μg per ml. This mean value was for Holstein milk, and the breed comparison, while probably not very significant, suggested that the milk of other breeds has less nicotinic acid than does Holstein milk. We are not inclined to question the accuracy of the figures of others, or of our own, since the assay method is so entirely satisfactory. The individual variation between cows (see page 79) seems the most likely explanation for somewhat low values, especially since, among eight samples of mixed market milks assayed during the preliminary study of the method, the range of values found was from 0.8 to 1.04 μg per ml with a mean value of 0.9. The bulk of this study concerned a large number of samples from a small number of cows, while values usually given in the literature have been for very few samples of market milk each representing a comparatively large number of cows. The average calcium pantothenate level for all milk samples was about 3.5 μg per ml, a value fairly typical of those reported heretofore. The average biotin level was about 20 μg per liter. The individual cows dealt with in this study may have been poor biotin producers, as six samples of mixed market milk ranged from 20 to 29 μg per liter and averaged 25 μg per liter.

The great variability among individual cows in the biotin content of the milk was very surprising, especially as it seems probable that a major source of this vitamin is rumen synthesis. Various workers (e.g., Lardinois et al., '44) have reported such synthesis. It is interesting that riboflavin in milk has also been found to show much variation, both between individual cows (Hand and Sharp, '39; Johnson, Maynard and Loosli, '41; Theophilus and Stamberg, '45), and from day to day from a single cow (Riddell, '37; Whitnah, Kunerth and Kramer, '38).

The seasonal change in the nicotinic acid level of the milk seems to be at least partly correlated with pasture feeding. No sudden marked changes occurred when cows went on or off pasture, but a trend in the direction of decreasing values was reversed when pasture feeding began, and again set in, when it was stopped. It seems likely that the effect of pasturage is not due to its nicotinic acid content. Nicotinic acid has been found to be among the B vitamins synthesized in the bovine rumen (Wegner et al., '40; Lardinois et al., '44), but changes in the rumen flora as a result of going on pasture would be expected to take place much more quickly, than the changes in nicotinic acid level revealed here.

There seems to be no doubt that the nicotinic acid, biotin, and pantothenic acid originally present in the milk, remains present and available to the consumer in the milk he purchases. The stability of these vitamins in milk is in line with the results of Hodson ('45) who found no losses in the preparation of irradiated evaporated milk and of dried milks, except for a possible small loss in biotin in dried skim milk.

SUMMARY

Modifications of the usual assay methods for nicotinic acid, biotin, and pantothenic acid, which were introduced in applying them to cow's milk samples, have been described. There was no evidence that a bound form of any of these vitamins exists in milk.

The biotin levels showed great variation among the milks of individual cows. Individual averages for eight cows ranged from 11 to 37 μg per liter. Between samples obtained from the same cow on successive days, the variation was also large. These variations were less marked in the case of nicotinic and pantothenic acids.

The nicotinic acid content was found to decrease during the winter and early spring months, and to increase during the summer and early fall months. The range of values on May 9 for eight cows was 0.22 to 0.77 μg per ml. On October 16 for five cows it was 0.56 to 0.96 μg per ml. A possible correlation with pasture feeding has been suggested. The biotin content had no apparent relation to the season.

The concentration of nicotinic acid in the milk decreased regularly through much of the lactation period. The pantothenic acid content of the colostrum at the time of freshening was found to be relatively low, the average level being about 1.5 μg calcium pantothenate per ml. The mean then rose during the next 9 days to a maximum of about 4 μg per ml, and then dropped to a normal level at about 3.5 μg per ml. A similar, though earlier, rise to a maximum and fall to normal tends to be true of the biotin level of milk.

The pantothenic acid and biotin contents of milk are correlated, though the nicotinic acid level appeared not to be correlated with either.

All three vitamins were found to be stable during pasteurization and exposure of the milk to sunlight.

ACKNOWLEDGMENT

We wish to express our appreciation for the help given us in this study by Dr. J. K. Loosli. The milk samples were obtained under his direction, and we have had his full cooperation in furnishing us with all necessary data pertaining to them.

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THE EFFECT OF LIVER EXTRACTS ON THE UTILIZATION OF CASEIN FOR GROWTH¹

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THREE FIGURES

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Liver has been shown to contain a factor, or factors, that has an accelerating effect upon the growth rate of rats and mice (Troscher-Elam and Evans, '41; McIntire et al., '43; Bosshardt et al., '45b). It has been suggested that a liver fraction may be necessary in order to obtain maximal utilization of certain dietary proteins in the growing rat. The results of Hayward and Hafner ('45) and Barnes ('45) showed an increase in the protein efficiency of a soyflour when Wilson's 1:20 liver concentrate or a butyl alcohol extract of this concentrate was included in the diet. No enhancement was noted when casein was used as the test-protein. Both proteins were fed at a level of approximately 10% in the diet.

The basal diet used by Bosshardt et al. ('45c) for the determination of the growth promoting quality of proteins included 1% of Wilson's 1:20 liver concentrate powder as an additional vitamin supplement. Since the liver concentrate powder contains 7 to 8% nitrogen, it supplies an appreciable amount of non-test nitrogen to the diet. This may introduce a serious error when low levels of test protein are used. The present study was designed to investigate the

¹ A preliminary report of this investigation was presented before the Philadelphia section of the American Chemical Society June 13, 1945.

effect of 1:20 liver concentrate and a butyl alcohol extract of this concentrate on the utilization of dietary protein by the growing mouse.

EXPERIMENTAL METHOD

The utilization of dietary protein for growth was determined in the manner described by Bosshardt et al. ('45c). Male weanling mice² were used. They were selected after a 2-day standardizing period during which they were fed a purified diet that contained 15% extracted whole egg. During the experimental periods, the animals were housed in individual wire-bottom cages and were supplied food and water ad libitum. Blotting paper was placed below the cages to facilitate the recovery of spilled food and the collection of feces with a minimum of urine contamination. All studies were of 20 days duration. Daily measurements were made of weight changes and food consumption. The feces of each test-group were collected for the entire test-period and "true digestibilities" were determined by the method described by Bosshardt and Barnes ('45a). Body protein gains were determined by analyses for carcass nitrogen.

Eight levels of casein ranging from 3% to 40% were studied, using groups of seven mice with each three accessory vitamin supplementations: control with no supplement, 1% of Wilson's 1:20 liver concentrate powder, and 0.15% of a butyl alcohol extract of Wilson's 1:20 liver concentrate powder prepared according to the method of Conger and Elvehjem ('41) (equivalent to 1.2% of 1:20 powder).³ The remainder of each diet consisted of 25% hydrogenated cottonseed oil,⁴ 2% corn oil,⁵ 20% dextrose,⁶ 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellu flour, and sufficient white dextrin to make 100%. To each 100 gm of diet were added:

² Sharp and Dohme, Swiss-Webster strain.

³ We are grateful to Dr. C. E. Graham of the Wilson Laboratories who supplied this extract.

⁴ Primex.

⁵ Mazola.

⁶ Cerelese.

4 mg of alpha-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1, 4-naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 21.6 mg of inositol, and 200 mg of choline chloride.

The effect on growth

The average weight gains of the mice fed varying amounts of casein with the three supplementations for the 20-day test-periods are illustrated in figure 1. These data confirm the

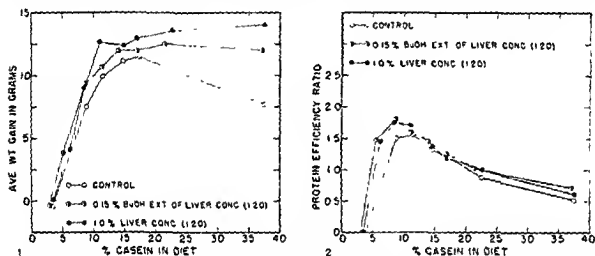


Fig. 1 The average 20-day weight gains of mice fed purified diets containing varying levels of casein with the three vitamin supplementations.

Fig. 2 The average protein efficiency ratios at 20 days determined with mice receiving purified diets containing varying levels of casein with the three vitamin supplementations.

reports previously mentioned that the addition of liver extract had an accelerating effect on the growth rate. This effect became more pronounced as the level of protein in the diet was increased.

The effect of the butyl alcohol extract was somewhat less marked than that of the original 1:20 liver concentrate. This may have been due to an incomplete extraction of the growth-accelerating factor by the butyl alcohol from the 1:20 liver concentrate.

The effect on protein utilization

Figure 2 illustrates the relationship between the level of casein in the diet and the protein efficiency ratio (gm gain in weight per gm of protein consumed) at the conclusion of a 20-day growth study. At the higher levels of protein, the protein efficiency ratio was not markedly increased by the two liver preparations, but it was consistently observed that maximal protein utilization was enhanced by both of these liver fractions. Additional studies have indicated that the protein efficiency ratio of casein is enhanced also by dried whole liver, defatted hog liver, and Valentine's ⁷ 1:50 liver concentrate. Data as presented in figure 2 are difficult to evaluate

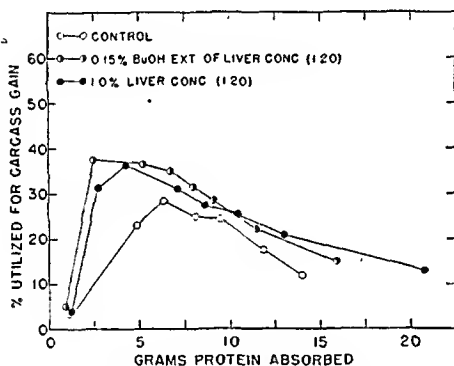


Fig. 3 The average percentage of absorbed protein utilized for body protein gain by mice during a 20-day growth study with varying intakes of casein with the three vitamin supplementations.

since no allowance is made for the differences in food consumption. Another possible source of error may be in the use of body weight gain as an index of body protein gain.

A more valid expression of the utilization of dietary protein for growth is the utilization of absorbed protein for body protein gain. This calculation was made as previously described by Bosshardt et al. ('45c). The results are shown in figure 3. A marked enhancement of protein utilization for growth by the two liver fractions was found at all levels

⁷ Valentine Meat Juice Co., Richmond, Va.

of protein intake, suggesting that liver contains a factor, or factors, of unknown composition that is essential for the maximal growth utilization of casein.

DISCUSSION

The data presented in a previous communication (Bosshardt et al., '45c) indicated that such factors as the pre-test standardization of the test animals, the duration of the feeding period, and the level of the test protein in the diet may have an influence on protein efficiency ratios. The results of the present study suggest an additional factor, dietary supplementation, that may affect the utilization of protein by the growing animal.

A purified diet containing only the accessory growth factors that are recognized as chemical entities will allow growth of mice. As previously mentioned this growth may be accelerated if a liver or pancreas fraction is included with the purified vitamin mixture. This enhancement of growth may be due either to a stimulation of the animal's appetite or to a more efficient utilization of the dietary ingredients or to a combination of both.

When a single level of protein in the diet is employed, differences in food consumption and growth are not considered adequately in the calculation of the protein efficiency ratio. Such differences are minimized if protein utilization is expressed either as the maximal ratio of body weight gained to protein consumed or as the maximal ratio of body protein gained to protein absorbed. The inability of other investigators to note an increase in the protein efficiency of casein with the inclusion of liver extracts in the diet may have been due to the fact that single levels of protein in the diet were compared and maximal ratios were not established. Furthermore, as is seen in figure 3, the effect of the liver extracts is more marked when protein utilization is expressed as body protein gain rather than as body weight gain. In fact at the level of the maximal ratio the inclusion of the liver extracts

resulted in an increase of approximately 30% in the protein utilization for growth.

It was found that the rate of growth as measured by body weight gain was enhanced by both liver preparations, but the effect was more marked in the case of the untreated preparation (Wilson's 1:20). In addition there was a definite increase in the utilization of the dietary protein for growth as measured both by the gm gain in body weight per gm of protein ingested and by the percentage utilization of absorbed protein for body protein gain. The two types of liver supplement appeared to be essentially equivalent in their enhancement of protein utilization as measured by these two methods. This study demonstrates the ability of liver extracts to increase the rate of growth and to increase the utilization of protein for body protein gain. The observation that these two functions were not affected to the same extent by the two types of liver extract suggests that two factors may have been involved.

SUMMARY

Two liver fractions, Wilson's 1:20 powder and a butyl alcohol extract of the 1:20 powder, have been shown to contain a factor, or factors, that enhances the growth rate and the growth utilization of dietary protein in mice. The effect on protein utilization is demonstrated most clearly when the maximal ratios of body protein gained to protein absorbed are compared.

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THE USE OF THE DOG FOR STUDIES ON IRON AVAILABILITY¹

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TWO FIGURES

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Dietary studies on the relation between the antianemic potency of various foods and their iron content when fed in the presence of adequate copper, have been made by numerous workers. The rat has been used almost exclusively in these investigations and consequently the applicability of these results to human nutrition may be open to some question. Since clinical investigations of this type are difficult, it follows that the work should be repeated with other species.

The dog was chosen for this study, and attempts were made to determine the availability of iron in several foods for the dog, and to determine the differences if any that exist between these values and those reported for the rat.

METHODS

Our first problem was to determine the minimal level at which the iron and iron containing foods should be fed to produce optimal hemoglobin formation. This would prevent the accumulation of large quantities of iron in the various tissues of the animal.

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To determine this level of iron, a litter of 5 collie puppies (1-6) and later a litter of 5 spaniel puppies (7-11) were placed on experiment. As in previous iron and copper work (Maass et al., '44) a diet of raw whole milk supplemented with vitamins and minerals, was fed *ad libitum*. That this diet is complete for the dog was demonstrated by Potter et al. ('38) who maintained dogs for long periods of time on a whole milk ration supplemented only with vitamins A and D together with iron, copper and manganese. If the proper precautions are taken against contamination, the amount of iron supplied by the diet is found to be very low (Frost et al., '40a). However, as a safeguard, the iron content of the milk fed was determined at frequent intervals (Ruegamer et al., '45).

Iron was supplied as ferric pyrophosphate.² This iron salt was selected because it is stable in solution and the iron is completely available. This salt together with the other mineral supplements, was added to a small amount of milk at each morning feeding.

Blood for analysis was removed from the radial vein; all samples being collected before the morning feeding. The external jugular and later the saphenous artery were used for phlebotomy when it became necessary to render the animals anemic. From 25 to 45% of the total blood volume (calculated as 8% body weight) was removed at each bleeding without apparent injury to the animals. One animal was later sacrificed for histological study and only mild hyperplasia of the bone marrow was noted.

Records were kept of the growth, the hemoglobin levels and the amount of iron fed. From these data, the total hemoglobin in the dog, hemoglobin made, iron used and the percentage of iron utilized were calculated (Frost et al., '40a). The percentage of availability of the iron in the test material can be calculated by assuming the inorganic iron to be 100% available. It should be noted that the iron furnished by the milk was not considered in the availability calculations since this factor remained constant throughout the experiment. In all

² Mallinckrodt, N. F. VI.

calculations, the total blood volume was considered to be 8% of the body weight. As shown by Hahn et al. ('42), the total blood volume of the dog is maintained at a constant level independent of the state of anemia.

The amount of plasma iron was determined routinely by the improved method of Kitzes et al. ('44) in an attempt to correlate the plasma iron levels with hemoglobin formation. Moore et al. ('37) state that iron is transported as plasma iron and that the quantity present in the peripheral blood is influenced by and is a measure of the amount of iron being absorbed from the gastro-intestinal tract, the iron reserve and various other factors.

RESULTS

The positive control (dog 1) from the litter of collie puppies was given 3 mg of iron per kg of body weight per day in addition to the regular mineral supplements and showed normal growth and hemoglobin levels throughout the experimental period. The remaining 5 dogs which were placed on the experimental diet at weaning, developed a severe anemia (Hb 2.1 to 3.8 gm %) in 4 to 5 weeks. At the end of the fifth week, dog 2 was given 200 μ g, dogs 3 and 4 400 μ g, and dogs 5 and 6 800 μ g of iron per kg of body weight per day. At the end of the first week of iron therapy, dogs 2 and 3 showed no hemoglobin response. Therefore, the level of iron for these animals was raised to 600 μ g of iron per kg of body weight per day. A rapid hemoglobin response occurred, suggesting that 600 μ g might be adequate for growing dogs. Dog 4 continued to make hemoglobin throughout the entire experiment even though confined to the lower level of 400 μ g of iron. Dogs 5 and 6, which were kept on 800 μ g, continued to make hemoglobin rapidly, showing a hemoglobin increase from 4.2 and 2.1 at the start of the experiment to 9.1 and 7.5 gm % at the end of the 6-week period. To determine whether the 600 or the 800 μ g level was most efficient for hemoglobin building, the per cent utilization for the iron was calculated and the results tabulated in table 1. At the end of the 6-week period.

the iron level of dogs 2 and 3 was raised to 1000 μg per kg of body weight per day. The per cent utilization for this level may also be found in table 1.

It was found that at a level of 600 μg of iron per kg per day (dogs 2 and 3) the utilization of the iron supplied as ferric pyrophosphate for hemoglobin building was 60 to 71%. Hemoglobin curves for the animals (dogs 2 and 3) receiving 600 μg of iron per kg of body weight per day showed approximately

TABLE 1

Utilization of ferric pyrophosphate for hemoglobin building over a 6-week period.

DOG NO.	LEVEL FED	IRON USED	IRON FED	IRON
	$\mu\text{g/kg/day}$	mg	mg	%
2	600	127	212	60
2	1000	210	541	39
3	600	134	189	71
3	1000	161	474	34
4	400	127	173	74
5	800	272	379	72
6	800	166	269	61
7	600	330	483	69
9	600	254	502	51
10	600	233	358	65
11	600	234	415	56

the same slope as that for the positive control (dog 1), receiving 3 mg of iron per kg per day (fig. 1). Levels of 800 μg (dogs 5 and 6) gave utilization of iron varying from 61 to 72% and levels of 1000 μg (dogs 2 and 3) caused the utilization to drop to 39 to 34% as would be expected. Dog 4 averaged 74% utilization on a level of 400 μg even though dog 3 failed to make hemoglobin at this level. Therefore, it can be concluded that the level of iron necessary for optimal hemoglobin building falls between 600 and 800 μg per kg of body weight per day.

From the data as plotted in figure 1, there appears to be a definite relationship between the intake of iron and the amount of iron in the plasma. When iron in excess of that

required for optimal hemoglobin formation is fed, the amount of iron in the plasma is increased. Thus when levels exceeding 600 μg of iron are fed, "normal" plasma iron values of 100-200 μg of iron per 100 ml plasma are found, and when levels below 600 μg of iron are fed, the plasma iron level drops to and sometimes below the critical level of 50 μg of iron per 100 ml of plasma. Since hemoglobin formation is greatly reduced when less than 600 μg of iron are fed, it may be concluded

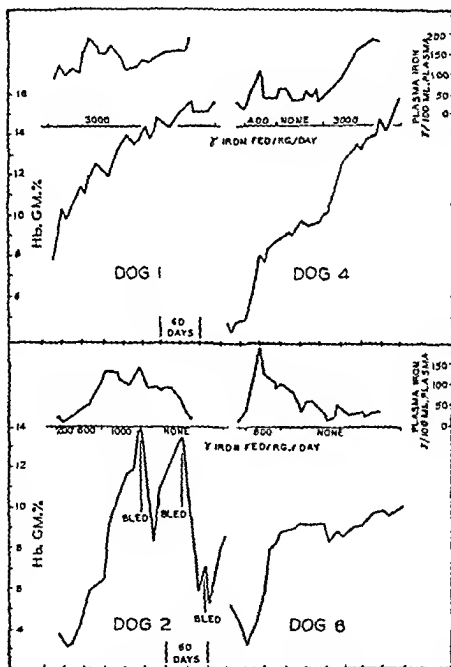


Fig. 1 Hemoglobin and plasma iron curves for dogs receiving different levels of iron as pyrophosphate.

that hemoglobin production is limited when the plasma iron level drops below 50 μg per 100 ml of plasma.

To verify our original conclusions, dogs 7-11 with the exception of dog 8 which served as a negative control, were given 600 μg of iron per kg of body weight per day after having been made anemic on a whole milk ration. At the end of 6 weeks, it was found that the animals were utilizing between 51 and 68% of the iron for hemoglobin building (table 1). These results agree with those obtained with the first litter of dogs, considering the breed difference and extent of individual variation. Therefore, it was decided to feed several biological materials at a level such that they would furnish 600 μg of iron per kg of body weight per day.

Wheat bran was selected as the first material to be fed because of its relatively high iron content, and palatability. For the experiment, the same 5 spaniel dogs were used. They were rendered anemic (hemoglobin 5-7 gm %) by phlebotomy. Between 30 and 40% of the total blood volume was removed at a single bleeding so as to render the animals anemic as quickly as possible. Once depleted of their iron reserves as evidenced by a plateau in the hemoglobin curves, dog 7 was chosen as a positive control and received 600 μg of iron as pyrophosphate per kg of body weight per day. Dog 8 served as a negative control and received no iron while dogs 9, 10 and 11 each received iron at a level of 600 μg per kg of body weight per day in the form of wheat bran.

The iron content of the bran was determined by ashing a 1 gm sample, dissolving the ash in dilute hydrochloric acid (1 part hydrochloric acid to 1 part water) and neutralizing and buffering the solution to a pH of 4.58. An aliquot was withdrawn, a reducing agent (thioglycolic acid) was added, and the amount of iron present was determined in an Evelyn Colorimeter with the addition of α - α -dipyridyl. A total of 16 samples of wheat bran were analyzed and the iron content found to be 11.5 mg/100 gm of bran, with a deviation of $\pm 2\%$. Since the animals weighed between 13 and 17 kg, between 70 and 100 gm of bran were fed daily to each dog. This supple-

ment was mixed with a little milk at each morning feeding, and the dogs were watched closely until the mixture was consumed.

At the end of the 6-week period, it was found that the utilization of iron as supplied by the bran and as fed as pyrophosphate, were approximately the same (table 2). Likewise, the slopes of the hemoglobin curves for dogs receiving bran (9, 10 and 11) were equivalent to the slope of the curve for

TABLE 2

Iron utilization for dogs receiving bran, spinach or ferric pyrophosphate as a source of iron over a 6-week period.

SOURCE	DOG NO	LEVEL FED <i>mg/kg/day</i>	IRON USED <i>mg</i>	IRON FED <i>mg</i>	IRON UTILIZED <i>%</i>
Bran	7 ¹	600	209	406	51
	9	600	259	385	68
	10	600	209	330	69
	11	600	259	429	60
Spinach	7	600	30	266	11
	9	600	51	274	18
	10	600	41	216	19
	11 ¹	600	134	260	51
Ferric pyrophosphate	7	600	303	516	59
	8	600	293	458	65
	9	600	248	367	68
	10	600	214	409	52
	11	600	151	337	45

¹ Control dogs receiving iron as ferric pyrophosphate.

the dog receiving iron (fig. 2). During this time, the negative control (dog 8) failed to make any significant amount of hemoglobin.

The supplements were discontinued and the dogs were rendered anemic by phlebotomy and maintained at this level for 3 or 4 weeks to make certain that the iron stores were depleted. Spinach was then fed as a supplement. The spinach was washed very carefully and dried at 46°C. by passing a stream of hot air over the material. The dried spinach was ground and the iron content determined as in the case of the

bran. 20.7 mg of iron per 100 gm of dried material were found, thus necessitating the feeding of approximately 40-50 gm of spinach to each dog daily. However, since the spinach proved to be unpalatable, it became necessary to mix the spinach with a small amount of milk at each feeding and give it by stomach tube. In this experiment, dog 11 received inorganic iron and served as the positive control and dogs 7,

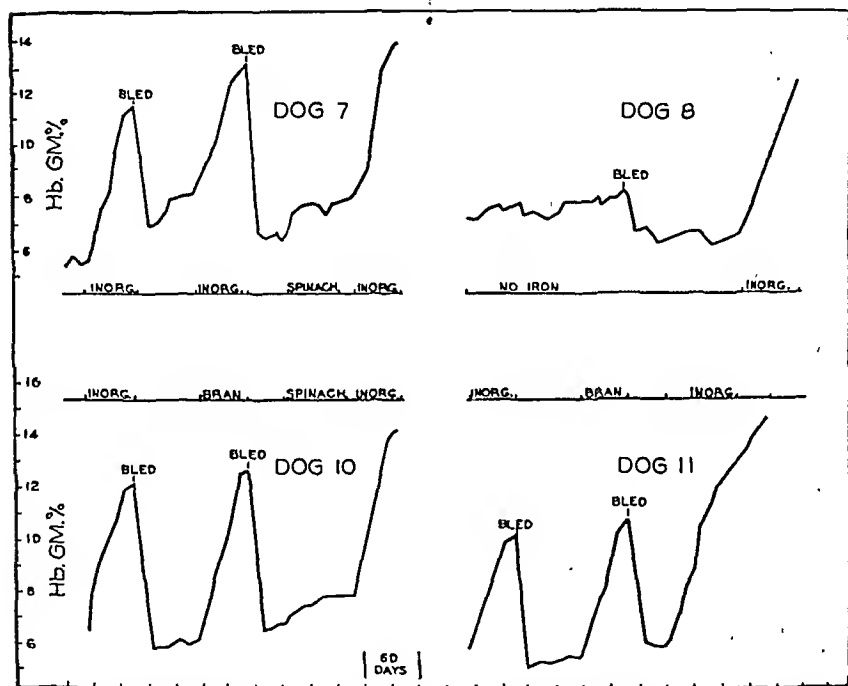


Fig. 2 Hemoglobin curves for dogs receiving iron as wheat bran, spinach and pyrophosphate.

9 and 10 received the spinach. Dog 8 served as the negative control as before. At the end of a 6-week period it was found that the spinach dogs (7, 9 and 10) utilized only 10 to 20% of the iron in the spinach for hemoglobin building, whereas the positive control (dog 11) utilized 50% (table 2). As can be seen from figure 2, there is also a marked difference in the slopes of the curves showing hemoglobin regeneration.

To determine if phlebotomy had had any effect on the blood building mechanism, all animals were given iron as pyrophosphate at a level of 600 μg per kg of body weight per day. As before, the animals utilized between 52 and 68% of the iron for hematopoiesis (table 2).

DISCUSSION

The results of this experiment indicate that levels of 600 to 800 μg of iron per kg of body weight per day are optimal for hemoglobin formation in the dog. Breed difference seems to have little or no effect on the iron requirement since it was possible to obtain essentially the same results in two breeds of dogs. If less than 600 μg of iron are fed, the animals will fail to make hemoglobin, and if more than 800 μg are fed, the per cent utilization will decrease. Therefore, since storage of iron in the tissues is to be avoided, the test materials were fed at a level such that 600 μg of iron were supplied per kg of body weight per day. That little storage of iron took place is shown by the hemoglobin curves (fig. 2). In each case, when rendered anemic, usually by one bleeding, the animal failed to make hemoglobin until iron supplementation was started.

The iron in bran was found to be almost completely available for hemoglobin formation. Of course, there is the possibility that additional factors supplied by the bran stimulated hemoglobin production. This seems unlikely however, since it has been demonstrated that milk will support hematopoiesis in dogs without the addition of factors other than iron, copper and manganese (Maass et al., '44; Frost et al., '40a, b).

When spinaeh was fed as the sole source of iron, very poor hemoglobin regeneration occurred. The per cent utilization of the spinaeh iron averaged between 10 and 20% as compared to 50% for the iron given as ferrie pyrophosphate. As mentioned previously, the spinaeh was dried at 46°C. to simplify the feeding problem and this processing might possibly have had some effect on the availability of the iron, since it has

been found that refrigeration, for example, will increase the availability of the iron in spinach (Hastings et al., '41). Under the conditions of this experiment, however, we found the availability of iron in spinach to be very poor.

Little iron contamination of the basal milk ration occurred as evidenced by the failure of the negative control to produce significant amounts of hemoglobin, and by the frequent assays of milk fed. However, one animal (dog 4) did make hemoglobin on the low level of 400 μg of iron per kg of body weight per day, and thus it is possible that this animal obtained iron from outside sources either from the cage or through handling.

When the values for the availability of iron in bran and spinach as obtained with this assay method on dogs are compared to those obtained with rats, a close correlation is found. Recent work in this laboratory with the rat has shown that the iron in bran is completely available as compared to ferric pyrophosphate (unpublished data). Sherman et al. ('34) found the iron in spinach as assayed by the α - α -bipyridine method and also the rat biological method to be only 20% available.

SUMMARY

Young growing dogs were placed on a raw whole milk ration, supplemented with vitamins, copper and manganese. When the dogs were anemic, supplements of ferric pyrophosphate at levels ranging from 200 μg to 1000 μg of iron per kg of body weight per day were supplied. A minimal level of 600 μg of iron was found to give an optimal hemoglobin response.

Plasma iron levels were followed throughout the period of iron supplementation, and it was found that when iron in excess of that required for optimal hemoglobin formation was fed, the amount of iron in the plasma increased. If sub-optimal amounts of iron were fed, the plasma iron level dropped to and sometimes below the critical level of 50 μg of iron per 100 ml plasma.

Wheat bran and spinach were fed at a level to supply 600 μ g of iron per kg of body weight per day, and the response compared with that obtained with ferric pyrophosphate. The iron in bran was found to be almost completely available while the iron in spinach was only 20-40% available.

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SELF SELECTION OF DIET

II. THE EFFECT OF FLAVOR¹

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From human experience, one could conclude that choice of food is to a large extent determined by flavor, and thus flavor of food may be important in determining nutritional status. Further, it has been often assumed that if animals refuse to eat a particular food, the taste of that food is unpleasant to the animals and a more pleasing flavor would be preferred. Hausmann ('33) reported that rats preferred to drink water flavored with saccharine to pure water. Jukes ('38) found that chickens avoided diets containing more than 2% salt, 0.03% quinine or 2% citric acid. Diets to which diacetyl was added were preferred by rats, according to Deuel and Movitt ('44).

The present experiments were designed to determine the importance of certain flavors in the self selection of diet by rats. A method of determining dietary preference for a single factor is described which is adaptable to a wide variety of dietary components.

METHODS

In the first experiment here reported, the eating behavior of animals allowed to choose from two identical diets was determined over a 6-week period to serve as a control test

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of the method. Ten male and ten female rats were weaned at 21-25 days of age. Each rat was placed alone in a cage with a rack containing two cups filled with a standard diet.² Vitamins were supplied separately as pills.³ The amount of food eaten from each cup was recorded daily and the cups were then alternated in a predetermined random sequence.

In testing for appetites for flavors, a small amount of flavor was added to the standard diet. The flavors used were diacetyl, 3 p.p.m.; oil of anise, 40 p.p.m.; monosodium glutamate, 1%⁴; butyric acid, 600 p.p.m., and trimethylamine in a not readily measurable amount.⁵ The flavors of the diets containing diacetyl and monosodium glutamate were quite mild, while the other three were offensively strong by human standards.

Ten weanling animals in each flavor test (five males and five females) were given the unflavored standard diet during a 3-week period in both cups, while another ten were given the flavored diet in both cups. At the end of this control period, both groups were allowed their choice of the standard and flavored diets for another 3 weeks. The amount of food eaten from each cup was recorded daily during both periods, and the cups alternated in the same predetermined random manner.

RESULTS

The results of the control experiment are shown in table 1. The 6-week period was divided in half to conform to the conditions of the other experiments, but very little difference

² The standard diet consisted of 24% purified casein (Labco "Vitamin-Free"), 10% hydrogenated fat (Primex), 4% salts (Joues and Foster, '42), and 62% sucrose.

³ Each pill contained approximately: 60 μ g thiamine hydrochloride; 120 μ g riboflavin; 90 μ g pyridoxine hydrochloride; 150 μ g calcium pantothenate; 10 mg choline chloride; 1 mg α -tocopherol and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola in a dextrin-powdered sugar base. One pill was fed to each animal daily.

⁴ The diet containing 1% monosodium glutamate contained 23% casein.

⁵ Trimethylamine gas was dissolved in a few ml of water, and the solution added to the diet until a strong fishy odor was apparent.

TABLE 1

Eating behavior of rats when no choice is offered.

FIRST 3 WEEKS ¹				SECOND 3 WEEKS				
	Wt gain	Total food eaten	% of food from		Wt gain	Total food eaten	% of food from	
			Left cup	Cup I ²			Left cup	Cup I
	gm	gm			gm	gm		
Males	59.2 ± 4.4	138.5 ± 9.4	48.1 ± 3.0	48.8 ± 3.1	61.4 ± 4.5	222.7 ± 16.2	49.4 ± 2.8	43.4 ± 4.0
Females	47.0 ± 2.4	123.0 ± 6.2	47.4 ± 2.4	49.5 ± 1.5	41.4 ± 2.3	184.7 ± 6.8	53.7 ± 4.4	54.4 ± 2.0

¹ All data in terms of mean and standard error of the mean.² Cup I was alternated between the left and right positions.

TABLE 2

Effect of diacetyl flavor on selection.

CONTROL PERIOD				EXPERIMENTAL PERIOD					
Diet	Wt gain	Total food eaten	% of food from		Diet	Wt. gain	Total food eaten	% of food from	
			Left cup	Cup I ¹				Left cup	Cup I
Group A Standard	51.1 ± 3.5	122.7 ± 5.3	53.7 ± 5.5	48.2 ± 7.3	Choice	64.8 ± 5.7	206.6 ± 10.9	50.9 ± 3.1	46.3 ± 8.6
Group B Flavored	58.3 ± 3.6	133.9 ± 7.3	43.5 ± 2.9	52.3 ± 3.6	Choice	65.5 ± 6.1	202.8 ± 14.2	44.2 ± 1.8	44.1 ± 5.2

Change in % eaten (control minus experimental)

	From left cup
Group A	2.8 ± 5.2
Group B	- 3.7 ± 4.0
All	- 0.4 ± 3.3

¹ Cup I contained the standard diet in the experimental period; cup II the diacetyl-flavored diet. A positive change in percent eaten from cup I (control minus experimental) therefore represents a preference for the flavor.

of the method. Ten male and ten female rats were weaned at 21-25 days of age. Each rat was placed alone in a cage with a rack containing two cups filled with a standard diet.² Vitamins were supplied separately as pills.³ The amount of food eaten from each cup was recorded daily and the cups were then alternated in a predetermined random sequence.

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⁴ The diet containing 1% monosodium glutamate contained 23% casein.

⁵ Trimethylamine gas was dissolved in a few ml of water, and the solution added to the diet until a strong fishy odor was apparent.

was observed in the two periods. Table 2 presents in detail the results of the experiment on diacetyl. The results with other flavors are shown in condensed form in table 3. The animals as a whole showed neither likes nor dislikes for any of the flavors, although the animals on an unflavored diet appeared to dislike trimethylamine (fishy) flavor.

DISCUSSION

The method of determination of dietary preferences described here is intended to be of general applicability. To adequately assess its usefulness, it is necessary to consider certain factors in animal behavior and mechanics of methods.

1. *Position eating.* In a 2-choice experiment many animals prefer to eat from either the left or right cup, and of these, a slight majority prefer the cup in the right-hand position. This habit was corrected for by alternating the cups. Since rats can learn a simple alternation pattern, the interchange was made random. The method of alternation was predetermined and was made identical in every case. In all experiments in this laboratory there have been no significant differences in the "position eating" habits of a group of rats when the control and experimental periods were compared. Tables 1 and 2 present typical cases.

2. *Repetitious eating.* If identical diets are offered, rats tend to eat from the cup they have eaten from before regardless of the change of position (Scott, '46). If the diets are not identical, repetitious eating by a single animal is indistinguishable from choice by a group because in repetitious eating both of the choices are eaten by the group in nearly equal amounts, while one is preferred if the group has made a choice. The present method then is valid only for demonstrating an appetite by a group.

When the diets are not identical, rats show a marked tendency to eat one diet or the other, but not both. This is indicated by the high values of the standard error of the mean of the change in per cent eaten from cup I in tables 2 and 3, and may easily be due to the fact that detectable dif-

Erratum

In the paper entitled "The carotene content of Cuban foods" by Angulo et al. which appeared in the April, 1946, issue, the data for carotene content should be in " $\mu\text{g/gm}$ of material" instead of mg/gm of material." Please insert in table 1, column 4 (pp. 466-467) " μg " in place of " mg ."

SOME EFFECTS OF DIETARY OXALATE ON THE TEETH OF WHITE RATS¹

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TWO FIGURES

(Received for publication April 22, 1946)

INTRODUCTION

Previous work in our laboratories (Restarski, Gortner and McCay, '45a; Gortner, Restarski, Bieri and McCay, '45) has shown that the ingestion of solutions of various food acids (phosphoric, citric, lactic, sulfuric) at pH 2.6 caused considerable gross destruction of rat molars *in vivo* within 1 week. In general the different acid solutions behaved similarly, although there were some differences in the relative severity with which they attacked the teeth.

McClure ('43) first showed that when rats were allowed to consume certain fruit juices or commercial soft drinks, destruction of the enamel occurred. Recently he has reported (McClure and Ruzicka, '46) that citrate solutions, even at neutrality, are capable of producing this result by slowly converting the insoluble calcium salts of the enamel into a soluble calcium citrate complex. In extending our studies to include the effects of oxalic acid and its sodium salts on the

¹ The opinions and views set forth in this article are those of the writers and are not to be considered as reflecting the policies of the Navy Department.

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molars of rats we have obtained effects which are more or less the counterpart of what McClure noticed with citrate. Although oxalic acid is a moderately strong acid, the anion combines with calcium to yield a highly insoluble substance.

METHODS

For this study young white rats, caged singly, were used. They were about 6 to 8 weeks of age and weighed from 75 to 125 gm at the beginning of the experiment. The basal diet employed in most experiments was the open formula dog feed⁴ used in previous studies. All diets were fed ad libitum. When solutions were being tested the daily allowance per rat was 20 ml. All solutions contained 10% of sucrose. The tests usually extended over a 7-day period, although in some cases they were continued for 2 or more weeks when no marked effect on the teeth was observed in the shorter period. The same method as described previously (Restarski, Gortner and McCay, '45b) was used for preparing the jaws and evaluating the effect of the acid solutions on the molars. Sufficient animals were included in each experiment to permit reliable assessment of differences.

EXPERIMENTS AND RESULTS

Oxalic acid solutions

To obtain a solution of pH 2.6 corresponding to acids previously tested, 285 mg of anhydrous oxalic acid were added per liter of solution. Twelve rats were given this solution for variable periods of time: six were sacrificed after 1 week, four more after 2 weeks, and the remaining two animals were killed after 12 weeks. In no instance did the oxalic acid solution etch the teeth. Even when the concentration of oxalic acid was quadrupled (1.14 gm per liter) to give a solution of pH 2.15, no etching was noted on the teeth of any of the eight rats drinking this solution for 3 weeks. Instead a definite opaque deposit was observed on all surfaces of the

⁴GLF dog feed. Canandaigua, New York.

molars which had been exposed above the gum line. This calculus-like encrustation was obtained when oxalic acid or sodium oxalate was included in either the feed or drinking water, the thickness of the deposit varying roughly with the concentration of oxalate and the length of time of ingestion (fig. 1A).

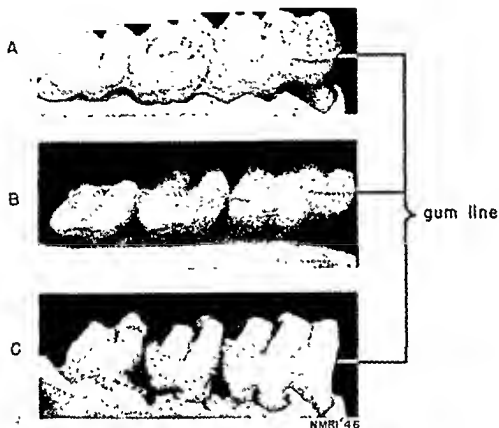


Fig. 1 Heavy calculus-like deposit on rat molars following ingestion of (A) a diet containing 50 mg per cent of sodium oxalate for 12 weeks, and (B) a diet containing 8% of dry spinach for 12 days, compared with normal rat molars (C).

*The protective action of oxalate against etching
by other acids*

It appeared logical that the characteristic film formed by oxalic acid ingestion was protecting the underlying enamel from acid attack. Accordingly, in order to learn whether oxalate might prevent *in vivo* tooth decalcification by other acids known to cause severe etching by themselves, a series of phosphoric and citric acid solutions was prepared and

molars of rats we have obtained effects which are more or less the counterpart of what McClure noticed with citrate. Although oxalic acid is a moderately strong acid, the anion combines with calcium to yield a highly insoluble substance.

METHODS

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⁴ GLF dog feed, Canandaigua, New York.

oxalic acid. After 1 week the animals were killed and their teeth examined and graded.

In all instances, even in the absence of oxalate, no appreciable etching of the upper molars was seen. This is consistent with observations previously reported (Gortner, Restarski, Bieri and McCay, '45) that citric acid differs from phosphoric acid in that it only mildly attacks the upper molars while severely damaging the lowers.

The data (fig. 2) show that increasing levels of oxalate afforded increasing protection of the mandibular molars up to about the level of 0.1% oxalic acid equivalent. Oxalate

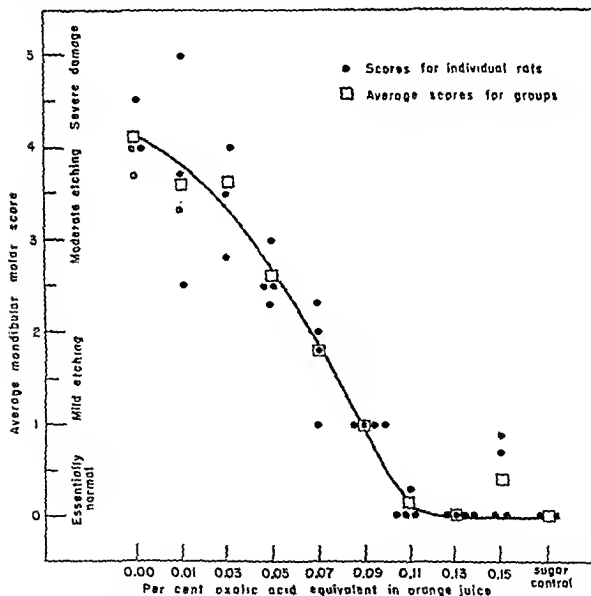


Fig. 2 The effect of different levels of soluble oxalate on the decalcifying action of orange juice on the teeth of rats.

concentrations above this percentage gave essentially complete protection against the orange juice and formed the opaque deposits on the teeth characteristic of oxalate ingestion.

Effects of some natural oxalate containing foods

Certain common foods, notably rhubarb and spinach, are known to contain appreciable levels of oxalate. Accordingly it was of interest to see whether these natural products would act on the teeth similarly to the oxalated feed and beverages studied above.

In the first attempt, equal weights of ground, dry dog feed and finely macerated, canned spinach or thawed, fresh-frozen rhubarb were thoroughly mixed and fed ad libitum to groups of rats over periods up to 4 weeks. No deposits resulted, however, on the molars of any of the animals. When they ingested a phosphoric acid solution of pH 2.6 for the last week of the experimental period, their teeth showed a degree of acid destruction comparable to that obtained with controls receiving the acid solution plus the dog feed alone (table 2).

TABLE 2

Effects of oxalate-containing foods, fed wet or dry, on the etching of rat teeth by a phosphoric acid-sucrose solution of pH 2.6, consumed for 1 week.

DIET	NO. OF RATS	AV. MOLAR SCORE	
Ground dog feed (basal)	12	3.6	} Moderately severe damage
Basal + spinach (wet)	8	3.7	
Basal + rhubarb (wet)	8	3.1	
Basal + 0.5% $\text{Na}_2\text{C}_2\text{O}_4$ (dry)	4	0.0	} No acid effect
Basal + spinach (dry)	8	0.0	

When, however, the spinach was previously dried before mixing with the ground dog feed diet (to give a mixture containing about 8% of dry spinach), and fed to a group of eight rats over a period of 12 days, the last week of which the

phosphoric acid leverage was also given, the results were as anticipated. A distinct enervation was found on all molars (fig. 1B) and no etching was apparent (table 2) when the deposit was scraped off and the tooth surfaces examined.

DISCUSSION

The foregoing results serve to show that moderate concentrations of soluble oxalate can effectively inhibit in vivo destruction of rat teeth by acids encountered in foods and beverages. The level of 0.1% oxalic acid (corresponding to a weekly intake per rat of 140 mg of oxalic acid, or the free and combined oxalate in 25 gm of fresh spinach) completely prevented tooth etching by orange juice.

Similar protection against tooth erosion by acids is afforded by natural foods, such as spinach and rhubarb. The petioles of rhubarb are reported (Winton and Winton, '35) to contain the equivalent of about 0.5% oxalic acid, of which approximately three-fifths is in water soluble form. Spinach likewise contains appreciable oxalate in this form. The negative results obtained when these foods were mixed wet with the ground dog feed apparently resulted from the calcium in the feed combining with the oxalate of the spinach or rhubarb so that no soluble oxalate remained in the mixtures as fed. When previously dried before mixing with the stock feed, spinach gave positive oxalate effects on the teeth. Similar results have been obtained by allowing rats to drink rhubarb juice for 1 week. Other foods known to contain moderate amounts of oxalic acid include beet greens, Swiss chard, green beans, taro and cocoa (Winton and Winton, '35).

Buonocore and Bibby ('45), in studying the effect of various ions on the acid solubility of pulverized human enamel, observed that oxalate ion was comparable to fluoride ion in reducing enamel solubility in vitro. Apparently, however, this finding has not been extended.

An interesting aspect of the present research is the observation that soluble oxalates in the food or drink may, over a period of time, build up calculus-like deposits on the teeth

of animals. Presumably this material is crystalline calcium oxalate, resulting from an interaction between oxalate ions and calcium in the saliva. Although human dental calculus is considered to be largely a calcium phosphate complex, it is possible that food oxalate may enter into its composition to a greater or lesser extent.

It should be emphasized that these experiments have been set up to obtain clear-cut effects within a short period of time. The results, therefore, do not justify condemnation of acid foods and beverages for human consumption; indeed some, such as citrus fruits, are valuable dietary constituents, even though they may well have some long range influence on the dentition of man.

Observations of this nature, however, may throw some light on human dental problems, such as tooth erosion and, perhaps, caries formation. It is a general observation of dentists that caries are seldom found under calculus deposits. Furthermore the results already obtained suggest that, at practicable levels, oxalate is more effective than fluoride in preventing in vivo dissolution of enamel by acids. In accordance with these observations, studies are now underway to determine whether levels of dietary oxalate sufficiently low to minimize tooth deposits and interference with calcium utilization will materially protect against caries development in the cotton rat.

The observations of Jones, Larsen and Pritchard ('30) on dental disease in Hawaii are of interest in this regard. They reported that the old Hawaiians in the rural districts, who clung to their old diets consisting primarily of taro (poi) and fish, had excellent teeth, but that the substitution of refined foodstuffs (polished rice, patent flour, etc.) in the diet of the urban groups was followed by obvious dental deterioration. Since taro has been reported to contain about 0.49% free and combined oxalic acid (Winton and Winton, '35) and the old Hawaiians consumed 5 pounds or more of poi daily, it is obvious that the diet was unusually high in oxalate content. How much of this was in a soluble form is problematical.

It is recognized that oxalic acid in the diet may have some influence on the utilization of calcium. The extensive literature existing on the toxicity and metabolism of oxalate in various animals and man is, however, largely outside the scope of this report. Suffice it to say that if the oxalate intake is appreciably great and the dietary calcium level is low, growth and bone formation may be definitely depressed.

In some of our experiments, when rats were maintained on a low calcium diet⁵ (either with or without small amounts of added sodium oxalate) for periods of 1 to 4 months, their growth was poor and at autopsy their bones were found to be soft and weak. On the other hand, the addition of as much as 0.5-1.0% of sodium oxalate to the dog-feed diet did not noticeably affect the growth or bone strength of rats consuming this diet for 4 weeks, suggesting that the stock feed had at least a moderately high calcium content. This is consistent with the observation of Maekenzie and McCollum ('37) that the growth and bone ash of rats receiving a diet optimal in calcium, phosphorus and vitamin D were unaffected when 0.9% of potassium oxalate was fed over 10 weeks. Even 2.5% of the oxalate had no effect on growth or gross calcium excretion, and had little effect on the bone ash.

The recent work of Mitchell and Smith ('45), who studied the calcium balance of a group of seven women students receiving diets low or borderline in calcium (225 to 700 mg per day), showed that no deleterious effects could be detected when cocoa was fed up to levels of 35 gm daily. Since cocoa contains 0.5-0.6% oxalic acid, the highest level would correspond to 175 mg of oxalic acid daily. Others (Bonner et al., '38) have reported that even when 700 mg of oxalic acid were added daily to the diet of children receiving 800 to 1300 mg of food calcium, no adverse effect on the calcium utilization was apparent. Thus, it appears probable that certain foods containing moderate levels of soluble oxalate may be safely

⁵ Degerminated white corn meal, 76%; corn oil, 10%; dried brewer's yeast, 5%; corn germ, 5%; cod-liver-oil, 3%; salt (NaCl), 1%.

included in the human dietary at average levels of calcium intake.

SUMMARY

1. Small amounts of soluble oxalate in the food or drink produced a hard deposit, which grossly resembled human dental calculus, on the teeth of rats. The extent of this encrustation, which is presumably calcium oxalate, varied with the concentration of oxalate consumed and with the length of the experiment.

2. Because of this phenomenon, oxalic acid solutions having a pH as low as 2.1 did not, *in vivo*, etch the enamel of the teeth of rats, in contrast to the action of other common food acids (citric, lactic, phosphoric, sulfuric) at even higher pH levels.

3. When oxalic acid or its sodium salt was present in the rat's food or drink the decalcification of teeth, which accompanies ingestion of phosphoric (0.055%) and citric (0.20%) acid solutions, diminished or disappeared. Essentially complete protection against the etching action of orange juice was afforded by a concentration of 0.1% oxalate in the juice.

4. Natural oxalate-containing foods, such as spinach and rhubarb, when incorporated into the diet in a manner which did not remove the soluble oxalate prior to ingestion, produced the characteristic protective films on the molars of rats within 1 week.

5. Inasmuch as these experiments with rats were devised to give clearcut results within a short time, caution must be exercised in interpreting these data with respect to human practices. It is hoped, however, that further studies using foods with moderate levels of "soluble" oxalate may offer some insight into the dietary control of dental calculus and caries initiation.

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DEPLETION OF HEPATIC RESERVES OF VITAMIN A AND CAROTENE IN CATTLE¹

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TWO FIGURES

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Various investigators have reported on the time required to produce symptoms of vitamin-A deficiency in cattle on rations low in carotene. Dickson, Jones and Schmidt ('35) and Guilbert and Hart ('35) have shown that night blindness is one of the first readily detectable clinical manifestations of vitamin-A deficiency to appear in cattle. Halverson and Sherwood ('30) produced night blindness in steers in 88 days on a ration of cottonseed hulls and meal while dairy heifers of about 1 year of age, on the same ration, required from 150 to 250 days to produce night blindness. Mead and Regan ('31) produced signs of vitamin-A deficiency in calves in 1 to 3 months after changing the ration from whole milk and grain to one low in vitamin A. Riggs ('40) has shown that range cattle from 3 to 16 months of age, on a ration practically devoid of carotene, will develop night blindness in from 46 to 266 days. The variability in time required to produce night blindness was shown to be dependent upon the age of the animal and the nature of the carotene-deficient ration as well as the ration fed previously.

Little information is available concerning the rate of depletion of hepatic reserves of vitamin A and carotene in cattle

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during the period of depletion of the body reserves of these two constituents. Guilbert and Hart ('35) have reported on the body reserves of vitamin A and carotene in cattle which had access to rations containing an abundant amount of carotene, and also on cattle which had the body reserves of the two constituents depleted to such an extent as to result in night blindness. Guilbert et al. ('37) have reported minimum vitamin-A and carotene requirements for cattle using night blindness as a test for minimum requirement.

The present paper deals with the rate of depletion of initially large vitamin-A and carotene reserves in the livers of steers while in the feed lot during an experimental period of 166 days. Two dietary groups are involved. One group was maintained on a ration high in carotene and the other on a ration relatively low in carotene. The data presented herein were obtained in one phase of a study designed to determine the cause of liver abscess formation in cattle.

EXPERIMENTAL

The animals used in the present experiment consisted of 140 Hereford steers of approximately 18 months of age at the beginning of the experiment. The steers were selected from a single herd and placed in the feed lot on November 9, 1944. The steers had been on native grass pasture in Jackson County, Colorado, since the previous spring and for several weeks prior to being placed in the feed lot had received a protein supplement of about 1 lb. of cottonseed cake daily.

Twenty-two animals were slaughtered at the beginning of the experiment and the remaining animals divided into two dietary groups. One dietary group of 98 animals was placed on a fattening ration as shown in table 2. The carbohydrate content of the ration was gradually increased during the feeding period of 166 days. All ration constituents, except salt and mineral supplement, were hand fed. The second dietary group of twenty animals was placed on a maintenance ration as shown in table 2, all self fed. The corn silage and cottonseed meal were gradually decreased as ration con-

stituents until at the end of 40 days the ration consisted entirely of alfalfa hay, salt and mineral supplement. Records were kept on consumption of ration constituents for both dietary groups. Random lots of animals were slaughtered at approximately 40-day intervals as shown in table 1.

Samples of liver tissue were obtained at the time each animal was slaughtered. The samples were sealed in glass jars containing dry ice and kept frozen until analyzed. Vitamin A was determined colorimetrically (Carr and Price, '26; Davies, '33) by means of an Amineo type-F photoelectric colorimeter. Crystalline vitamin-A alcohol was used as the reference standard. The vitamin A and carotene were extracted

TABLE 1
Slaughtering data.

DAYS IN FEED LOT	NUMBER OF ANIMALS SLAUGHTERED	
	Fattening ration	Maintenance ration
0	22	
41	19	
76	20	
119	19	10
166	40	10

from the saponified liver with petroleum ether and dried over anhydrous sodium sulphate. The carotene was determined colorimetrically in the dried petroleum ether extract. Beta-carotene, dissolved in petroleum ether, was used to obtain the standard curve on the colorimeter.

The average values found for vitamin A and carotene for each group of animals slaughtered are shown in figure 1. One liver sample was lost from the group on the maintenance ration slaughtered after 166 days in the feed lot. Otherwise, the number of livers analyzed is as given in table 1.

The ration constituents were analyzed for carotene content at frequent intervals and the carotene intake per animal per day calculated. The values so obtained are summarized in

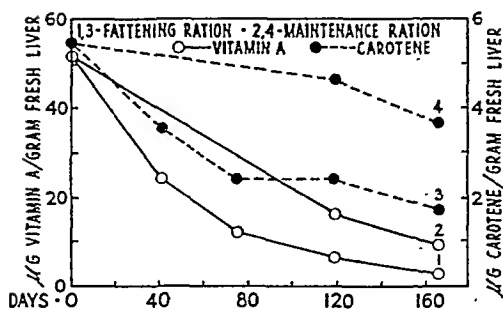


Fig. 1 Graphical representation of depletion of liver vitamin A and carotene reserves of steers with days in the feed lot. Expressed as micrograms of constituent per gm of fresh liver.

TABLE 2

Average daily ingestion of ration ingredients in pounds and the calculated carotene intake per animal.

FEEDING INTERVAL IN DAYS	ALFALFA HAY	CORN SILAGE	GROUND CORN	BRAN	COTTON SEED MEAL	CAROTENE, IN $\mu\text{G} \times 10^3$
Fattening ration						
0- 41	8.14	6.14	9.11	0.00	1.25	469
41- 76	4.89	5.01	14.98	0.72	1.00	317
76-119	3.76	3.72	16.63	0.96	0.95	237
119-166	3.17	2.69	18.27	0.99	0.98	205
Maintenance ration						
0- 41	15.96	6.60	0.00	0.00	0.50	883
41- 76	20.13	0.00	0.00	0.00	0.00	1030
76-119	17.23	0.00	0.00	0.00	0.00	878
119-166	20.07	0.00	0.00	0.00	0.00	1060

table 2, each value representing an average over a feeding period of approximately 40 days.

DISCUSSION

The data given herein include values obtained from both normal livers and those showing a pathological condition. The only pathological conditions found were telangiectasis, abscess and "sawdust." Barron ('42) has reported variations in the vitamin-A content of the liver as the result of various pathological conditions. Unpublished data by the present

authors show no significant variation in the vitamin-A content of the liver as a result of the pathological conditions observed in the animals under investigation.

A definite trend is shown in the vitamin-A and carotene values for the animals on the fattening ration throughout the entire feeding period of 166 days (fig. 1, curves 1 and 3). There is some indication of a similar trend in the vitamin-A values for the animals on the maintenance ration (fig. 1, curve 2). Depletion of initially large reserves of both constituents are indicated in the curves cited, the rate of depletion diminishing as the feeding period progressed. The carotene intake for the animals on the maintenance ration was relatively high compared to the carotene intake for the animals on the fattening ration (table 2). This would account for the high level of carotene reserve maintained by the animals on the maintenance ration (fig. 1, curve 4). The above is in agreement with the work of Baumann, Riising and Steenboek ('34) who showed that the rate of depletion of vitamin A in the liver of the rat decreases with time as the liver reserve of vitamin A becomes smaller.

The average amount of carotene ingested daily by the animals on the maintenance ration was 963 mg (table 2). The largest total liver reserve of carotene found in any of the experimental animals was 50 mg. This would indicate a daily requirement of carotene by cattle considerably in excess of the total hepatic storage in order to maintain the liver reserve of carotene.

No clinical evidence of avitaminosis A was observed in any of the animals during the experimental period. Consistent gains in weight were obtained for the animals in both dietary groups. Guilbert et al. ('37) obtained excellent gains in weight in cattle with a very low storage of vitamin A. Jones et al. ('38) found young Hereford steers to become very fat on a ration practically devoid of carotene even though the animals were totally night blind. The average vitamin-A value of the livers of the animals on the fattening ration, after 166 days in the feed lot, was 1.9 μ g of vitamin A per gm of fresh

liver (fig. 1). This would indicate that hepatic reserves of vitamin A may fall to an extremely low level in cattle without producing acute symptoms of avitaminosis A.

A comparison of the percentages of the hepatic reserves of vitamin A and carotene retained in each of the two dietary groups, after 166 days in the feed lot, shows that the carotene reserves of the liver are more readily maintained than are the vitamin A reserves (table 3). At the beginning of the experimental period there was present in the liver 9.68 μ g of

TABLE 3

Initial and final hepatic reserves of vitamin A and carotene (see fig. 1) expressed as micrograms of constituent per gram of liver.

INITIAL RESERVE	VITAMIN A	CAROTENE
	51.4	5.31
	Fattening ration	
Final reserve	1.9	1.80
Per cent of initial reserve	3.7	33.9
	Maintenance ration	
Final reserve	9.4	3.77
Per cent of initial reserve	18.3	71.0

vitamin A for each microgram of carotene (fig. 2). After 166 days in the feed lot this value had dropped to 1.06 μ g for the animals on the fattening ration and to 2.50 μ g for the animals on the maintenance ration. Braun ('45) reported vitamin-A and carotenoid values of the blood and liver of cattle with and without vitamin-A supplement with a view to establishing a relationship between vitamin-A levels of the blood and liver. When vitamin-A and carotenoid values for individual livers were compared he found the ratio $\frac{\text{vitamin A}}{\text{carotenoid}}$ to decrease with increasing carotenoid levels of the liver. Reference to figure 2 will show increasing values for the ratio $\frac{\text{vitamin A}}{\text{carotene}}$ of the liver with increasing carotene levels of the liver.

The values for the ratio $\frac{\text{carotene content of liver}}{\text{daily carotene intake}}$ show no significant variation (table 4). This would seem to indicate that the carotene reserves of the liver vary in direct proportion to the carotene intake.

The values for the ratio $\frac{\text{vitamin A content of liver}}{\text{daily carotene intake}}$ show successively decreasing values (table 4). The decreasing values for the ratio indicate an increasing rate of loss of hepatic reserves of vitamin A with decreasing carotene intake. Corresponding ratios for the animals on the maintenance ration are not given since the animals were on a fairly constant carotene intake.

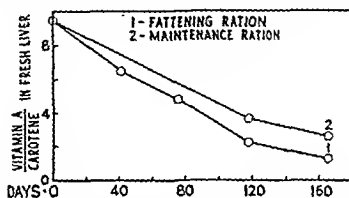


Fig. 2 Graphical representation of the decrease in the $\frac{\text{vitamin A}}{\text{carotene}}$ ratio of the liver with days in the feed lot. Expressed as micrograms of constituent per gm of fresh liver.

TABLE 4

The ratios $\frac{\text{carotene content of liver}^1}{\text{daily carotene intake}}$ and $\frac{\text{vitamin A content of liver}^1}{\text{daily carotene intake}}$ for the animals on the fattening ration. Daily carotene intake given in grams (table 2) and liver constituents in micrograms per gram of fresh liver.

FEEDING INTERVAL IN DAYS	CAROTENE CONTENT OF LIVER	VITAMIN A CONTENT OF LIVER
	DAILY CAROTENE INTAKE	DAILY CAROTENE INTAKE
0- 41	7.65	50.5
41- 76	7.66	37.5
76-119	10.00	22.4
119-166	8.78	9.3

¹ Values for carotene and vitamin A, as found at the end of the feeding interval, were used in calculating the above ratios.

SUMMARY

The rate of depletion of initially large hepatic reserves of vitamin A and carotene is given for 140 steers over an experimental period of 166 days. The animals were divided into two dietary groups. One group was on a fattening ration low

in carotene and the other on a maintenance ration relatively high in carotene. The rate of depletion of the hepatic reserves of vitamin-A and carotene decreased as the liver reserves of the two constituents decreased.

No clinical evidence of avitaminosis A was observed in any of the animals throughout the experimental period. This would indicate the possibility of extremely low levels of vitamin A reserve in cattle without producing symptoms of avitaminosis A.

Hepatic reserves of vitamin A were found to be more readily depleted than were hepatic reserves of carotene. Increasing values were found for the ratio $\frac{\text{vitamin A}}{\text{carotene}}$ of the liver with increasing carotene reserves of the liver. It was found that hepatic reserves of carotene are maintained in cattle in direct proportion to the carotene intake. An increasing rate of loss of hepatic reserves of vitamin A occurred with decreasing carotene intake.

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RIBOFLAVIN EXCRETIONS OF YOUNG WOMEN ON DIETS CONTAINING VARYING LEVELS OF THE B VITAMINS¹

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ONE FIGURE

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Although a number of studies have been made on the excretion of riboflavin by persons maintained on a controlled intake of the vitamin, only a few have had women as subjects. Sebrell, Butler, Wooley and Isbell ('41) maintained a group of ten women on a diet containing 0.21 mg riboflavin per 1000 Cal. for a period of 4 to 8 months. The daily excretion averaged 74 μ g riboflavin per day. Six of the women developed cheilosis. On the basis of added amounts of the vitamin required to prevent or cure cheilosis and of the urinary excretion on different levels of intake, these workers suggested that the riboflavin requirement was about 3 mg/day, or about 1.2 to 1.5 mg/1000 Cal. The results of a brief experiment on four college girls by Strong, Feeney, Moore and Parsons ('41) appeared to confirm this recommendation. On a dietary intake of 1 to 2 mg riboflavin the daily urinary excretion was 50 to 150 μ g, in comparison to a 500 to 800 μ g daily excretion observed in a group of students and faculty members eating

¹ The expenses of this study were defrayed by funds granted by the National Dairy Council on behalf of the American Dairy Association and by the Williams-Waterman Fund of the Research Corporation.

² The data reported in this paper were taken from a thesis presented by Margaret V. Davis to the Faculty of the Division of Biological Sciences of the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

their normal diet. More recent work indicates that these early recommendations were high, however. The daily urinary excretion of riboflavin and the response to a test dose of the vitamin by groups of women maintained on different levels of riboflavin intake were studied by Williams, Mason, Cusick and Wilder ('43). Urinary excretion fell to about 100 μ g/day and test dose returns were markedly lowered on an intake of 0.35 mg/1000 Cal. On an intake of 0.5 mg/1000 Cal., daily excretion fell to low levels but test dose returns were not affected as greatly as on the smaller amount. On an intake of 0.8 mg/1000 Cal. there was no noticeable decrease in the excretions. These workers suggested that the requirement of riboflavin by women was not less than 0.5 mg, nor more than 0.8 mg/1000 Cal., or a total of 1.0 to 1.6 mg per day.

In the present work the riboflavin excretion of a group of healthy young women, living on a diet in which the amount of riboflavin was progressively increased, was followed over a period of 8 months. The young women were university students, leading a life characteristic of such a group. Efforts were made to insure that the experimental procedures should result in as little change as possible in their usual habits. It was hoped that such a study would yield further information on the utilization of and requirement for riboflavin by normal young women, insofar as such requirements can be judged on the basis of excretion of the vitamin on known levels of intake. That the excretion level of a vitamin reflects the tissue concentration of that vitamin has been demonstrated in the case of thiamine by Hulse and co-workers ('44).

METHODS OF STUDY

Altogether twelve young women between the ages of 19 and 32 years served as subjects during the course of the study. Of the nine original subjects, three were followed throughout the entire 8-month period, the others for periods of $3\frac{1}{2}$ to 5 months. Three months before the end of the study it was decided that at the level of riboflavin intake planned for the remainder of the time, new subjects might be included after

a short period of adjustment to the diet. Three young women were selected to serve as subjects at this time, together with three who had been on the diet since the beginning.

Each subject received a complete physical and neurological examination some time during the first 2 weeks of the study.³ This examination was repeated after a period of 7 weeks on the experimental diet. At the end of 3½ months a special examination was made for any signs of riboflavin deficiency.

The results of the preliminary examinations showed the young women to be in a good general state of health. Two persons (subjects 7 and 10) were found to have hemoglobin values somewhat below normal, and three deviated considerably from the average weight for their height. Subject 2 was 17% overweight, while subjects 1 and 3 were approximately 20% underweight. There were no other negative findings.

The experiment was divided into five periods. During the first period, which lasted for 106 days, the average intake of riboflavin was 0.29 mg/1000 Cal. After the first 59 days on this level certain changes were made in the diet which increased the intake of thiamine, but did not change that of riboflavin. The times before and after this change have been designated as periods I-a and I-b. In period II, 45 days in length, the riboflavin intake was increased to an average of 0.49 mg/1000 Cal., and in period III, which lasted 41 days, to an average of 0.66 mg/1000 Cal. In period V, 14 days long, the diet was supplemented with 6 mg riboflavin, 4 mg thiamine, 0.5 mg pantothenic acid, and 10 mg niacin per day. This period of "superecharging" was followed by a return, for 20 days, to approximately the same level of intake as in period III.

Except during period IV, when the supplements of synthetic vitamins were given, and during periods III and V when a daily supplement of approximately 100 µg of thiamine was given, all variations in the vitamin content of the diet were made through changes in the kinds and amounts of foods.

³ Grateful acknowledgment is made to Dr. Oscar Kreisler, who kindly made these examinations.

This was done because it was considered that the results would be more applicable to practical situations than if the synthetic vitamin were used.

The menus for the experimental diet were worked out on the basis of 3-day periods. The same foods were served each 3 days, although their method of preparation was varied.

TABLE 1

Amounts of basic foods served every 3 days during each experimental period.

FOOD	PERIOD				FOOD	PERIOD			
	I-a	I-b	II	III and V		I-a	I-b	II	III and V ¹
	gm	gm	gm	gm		gm	gm	gm	gm
Beef	150	150	200	200	Rhubarb	100	.
Chicken	75	75	100	100	Berries	100
Carrots	70	70	100	100	Grapefruit				
Beets	70	70	juice	120	120
Green beans	60	60	100	100	Tomato juice	120	...	120	120
Peas	.	.	100	100	Apple juice	120
Potatoes	100	100	300	300	Orange juice	...	200	360	360
Head lettuce	100	100	190	210	Rice	200	200
Celery	30	30	20	...	Farina	340
Pineapple	72	72	200	200	Oatmeal	...	300	400	400
Apple	200	200	100	.	Cream	<132	<110	<110	<110
Peaches		...	100	100	Milk	600	1050 ²
Pears	.	.	.	100	Jam	<150	<150	<150	<150

¹ The same general dietary pattern was followed during period IV, when supplements were given, as during periods III and V. Some variation was allowed in period IV, however.

² In an attempt to keep vitamin/calorie ratios similar for all subjects, subject 2 received 600 gm of milk every 3 days, and subject 12, 900 gm every 3 days during periods III and V.

Foods which contained considerable amounts of riboflavin, such as meat, milk, fruits and vegetables, were served in equal quantities to each person. The amounts of these foods served in each 3-day period will be found in table 1.

In order to increase the caloric intake, cake and cookies and usually some form of pastry were served in each 3-day period in approximately equal amounts to each person. Bread and

butter were allowed ad libitum as required to meet individual caloric needs. All of the baked products were prepared in the laboratory using unenriched flour, low vitamin yeast, water, and minimal amounts of egg.

A complete record of food consumption was kept for each individual. No food was eaten except that served at meals, although outside consumption of certain beverages was permitted. A careful record of these was kept by each subject, and they were included when the caloric value of the diet was calculated.

The basal diet was planned to be low in both riboflavin and thiamine. The niacin content of the diet in the early periods was only slightly below the present recommended allowance of 12 mg per day for moderately active women. It was assumed that the diet was adequate in other B-vitamins for which requirements are not known. Supplements of di-calcium phosphate, ferric pyrophosphate, vitamin A and ascorbic acid were given. Complete details of the nutritive value of the diet and of its supplementation will be found in table 2. The amount of iron given in period I-b was increased over that in period I-a in connection with a study on serum iron which was made on the subjects during period I.

Preliminary observations on the average daily excretion of riboflavin, the fasting 1-hour excretion, and the return of a test dose were made during the week prior to the start of the experimental diet. The subjects continued on a self-chosen diet during this week, except that they were asked not to eat liver or pork during the period.

During the course of the experiment 72-hour urine collections were made each week, except during the first week of a new experimental period. The urine samples were collected in brown glass bottles, and preserved with acetic acid, toluene, and a few drops of chloroform. One-fifth of the entire 3-day sample was held under refrigeration until analyzed.

During the last week in each experimental period two 1-hour specimens of urine voided before breakfast were collected. On one morning during this week a low-vitamin breakfast

TABLE 2

Length of periods, number of subjects, and composition of the diet throughout the study.

	PERIOD					
	I-a	I-b	II	III	IV	V
Length (days)	59	47	45	41	14	20
Number of subjects	9	9	5	6	6	6
<i>Average intake¹</i>						
Riboflavin — mg/1000 Cal.	0.29	0.28	0.49	0.66	4.10 ⁴	0.63
Thiamine — mg/1000 Cal.	0.14	0.20	0.36	0.51 ²	2.80 ⁴	0.54 ²
Niacin — mg	11.0	9.7	10.6	14.3	F ² 14.3 S 10.0	14.3
Pantothenic acid — mg	2.3	2.4	3.5	4.3	F 4.3 S 0.5	4.7
Protein — gm	49	51	61	62	62	62
Fat — gm	73	78	82	68	68	68
Carbohydrate — gm	311	307	289	247	247	247
Vitamin A — I.U. ²	F 4200 S ..	F 4200 S ..	F 6300 S 5000	F 6500 S 5000	F 6500 S 5000	F 6500 S 5000
Ca — gm	F 0.17 S 0.35	F 0.22 S 0.24	F 0.47 S 0.24	F 0.67 S 0.24	F 0.67 S 0.24	F 0.67 S 0.24
Fe — mg	F 6.9 S 4.0	F 7.6 S 8.0	F 10.8 S ..	F 11.3 S ..	F 11.3 S ..	F 11.3 S ..
Vitamin C — mg	F42.4 S50.0	F70.8 S50.0	F113.4 S 25.0	F115.3 S 25.0	F115.3 S 25.0	F115.3 S 25.0

¹ Figures for riboflavin, thiamine, nicotinic acid and pantothenic acid determined by analysis; those for other nutrients by calculation.

² International Units.

² Figure includes supplement of approximately 100 µg thiamine per day.

⁴ Figure includes supplements — 6 mg riboflavin, 4 mg thiamine.

⁵ F — amount supplied by food; S — amount supplied by supplement.

was given, and the urine excreted during the following 4 hours was collected. This constituted a "4-hour control sample." On the next to last morning of each period a test dose of riboflavin was given orally with the same low-vitamin breakfast and a 4-hour sample collected.⁴ The size of the test dose was 0.02 mg/kg body weight, which gave a total of approximately 1 mg. In calculating the percentage of the test dose which was returned, the riboflavin value of the 4-hour control sample was subtracted from that of the 4-hour sample taken following the test dose.

During the times when urine collections were being made, samples of all foods were saved for analysis. Composites, representing one-fifth of the total amount of food taken by each person, with the exception of cereal foods, sugar, and butter, were made at the end of the 3-day periods. The samples were homogenized, brought to approximately pH 1 by the addition of 8 ml concentrated hydrochloric acid per liter of suspension, and autoclaved at 15 pounds pressure for 15 minutes. Cereal foods were analyzed separately and the amount of riboflavin taken by each person in such foods was determined by calculation. This procedure was followed in order that the cereals would not exert any untoward effects on the riboflavin analyses.

Fecal collections, marked by carmine, representing the same period as the last 3-day urine collection in each period except period IV were made by some of the subjects. After the wet weight had been recorded, the feces were placed in a large evaporating dish and covered with 50% alcohol. Succeeding samples were added to the dish, which was kept refrigerated until the collection was complete. The samples were dried to constant weight at 45°C. After drying they were ground and stored in the refrigerator until analyzed.

The riboflavin content of the food composites, urine, and feces was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42). A modified

⁴ Riboflavin was generously supplied by Merek and Company.

basal medium, the same as that used by Oldham, Johnston, Kleiger and Hedderich ('44) for cereal analyses, was used for all analyses in this study.

RESULTS AND DISCUSSION

A summary of the results of the study is found in table 3. Included in this table are the results of (1) the preliminary measurements of urinary excretion of riboflavin, (2) the average daily intake and urinary excretion of the vitamin by each subject during each period, and (3) the average daily fecal excretion, fasting 1-hour excretion and return of a test dose at the end of each period.

Results of preliminary observations. As would be expected among a group of people of different dietary habits, there was considerable variation in the daily urinary excretion of riboflavin during the preliminary period. There appeared to be a relationship between the daily excretion and the amounts of riboflavin-containing foods, particularly of milk, taken by the young women. Subjects 7 and 11, who had the lowest daily excretions, drank little milk. All of the others were accustomed to drinking milk in varying amounts.

Only four of the subjects returned as much as 20% of the test dose, the amount which was suggested by Oldham and co-workers ('44) as indicative, in the case of children, of a satisfactory nutritional status with regard to riboflavin. One of these four returned approximately 35%, which is the amount suggested by Feder, Lewis and Alden ('44) as a normal return of a test dose. It should be noted, however, that in the data presented by Feder et al., the total 4-hour excretion following the administration of the test dose is used in calculating the percentage returned. In the method of calculation used in this paper, where the normal 4-hour excretion is subtracted from the amount excreted in the 4 hours following the test dose, a smaller percentage return would be expected. Although the return of a test dose has been used as a criterion in nearly every study of riboflavin requirements, the size of the test dose, the method of its administration, and

of calculating the return have varied. Because of these variations it is difficult to assess the original nutritional status of the subjects on the basis of test dose returns.

Twenty-four-hour excretions

The 24-hour excretions of riboflavin adjusted to changes in intake quite rapidly. After about 10 days on a new dietary level, the daily excretions reached approximately the level at which they remained throughout the period. One exception was observed during period I-a. This was subject 2, whose excretion of riboflavin during the preliminary period was much higher than that of anyone else. Her riboflavin excretion dropped gradually during the first 6 weeks on the experimental diet and by the seventh week had reached a level at which it continued until the end of period I-b. In the case of subjects 7 and 11, who had shown low daily excretions of riboflavin during the preliminary period, the 24-hour excretion level did not change significantly when the experimental diet was started.

In spite of the relatively large increase in riboflavin intake in period II, from 0.29 to 0.49 mg/1000 Cal., there was only a small increase in the daily excretion of the vitamin. However, when the riboflavin intake was increased in period III to 0.66 mg/1000 Cal., there was a definite rise in excretion in all subjects. As would be expected, an immediate, striking rise in daily excretion occurred in period IV when the diet was supplemented with 6 mg riboflavin. Urine collections were made during the first 3 days and last 3 days of this period. An average of 55% of the total intake of riboflavin was excreted during the first 3 days, and of 66% during the last 3 days. Apparently there was a tendency for the body to "hold on" to some of the extra vitamin at first. Subject 2 excreted a greater proportion of the supplement than did the others; this indicates, perhaps, that her stores were more nearly full than were those of the other subjects.

Following the vitamin supplementation, and at the end of 2 weeks on an average daily intake of 0.63 mg/1000 Cal., the

TABLE 3

Average daily intake and excretion of riboflavin by each subject throughout the experiment together with average daily fecal excretion, fasting 1-hour excretion and test dose return by each subject at the end of each experimental period.

SUBJECT	1	2	3	4	5	6	7	8	9	10	11	12	WITH
HEIGHT, CM	165	169	161	170	160	152	164	175	165	158	174	170	170
WEIGHT, KG	46.3	71.7	45.8	56.2	56.2	50.8	58.5	62.6	59.0	59.4	63.5	69.4	69.4

Preliminary period													
<i>Excretion</i>													
Urinary μg/24 hr.	480	931	305	617	283	508	145	628	421	190	109	581	421
Fasting μg/hour	8	34	17	34	20	13	8	25	12	15	8	22	15
Test dose % return	13.3	34.8	20.2	13.0	15.9	23.1	11.8	..	8.2	13.1	10.6	19.6	17.7

Period I-a													
<i>Intake</i>													
Total, μg	631	555	539	561	608	...	658	671	610	...	633	...	633
Mg/1000 Cal.	0.29	0.33	0.27	0.30	0.29	...	0.29	0.27	0.29	...	0.27	...	0.27
<i>Excretion</i>													
Urinary μg/24 hr.	114	183	88	124	80	..	129	127	97	...	98	..	118
Faecal μg/24 hr.	478	598	872	..	794	633
Fasting μg/hour	7	11	7	6	7	..	7	8	8	...	6	..	8
Test dose % return	0.3	11.6	3.3	3.2	2.2	...	2.6	1.0	1.0	..	0.1	..	2.8

Period I-b													
<i>Intake</i>													
Total	585	529	534	572	523	..	649	629	566	...	625	..	585
Mg/1000 Cal.	0.27	0.30	0.23	0.27	0.29	..	0.27	0.27	0.27	...	0.26	..	0.25
<i>Excretion</i>													
Urinary μg/24 hr.	107	125	84	95	82	...	142	115	92	...	97	..	151
Faecal μg/24 hr.	407	399	573	512	587	463
Fasting μg/hour	6	8	8	4	4	..	4	5	6	..	6	...	6
Test dose % return	7.0	11.6	5.0	2.9	1.5	..	4.3	0.8	3.3	..	4.0	...	4.5

Period II													
<i>Intake</i>													
Total	1070	967	1013	1056	981	1017
Mg/1000 Cal.	0.48	0.57	0.47	0.44	0.51	0.45
<i>Excretion</i>													
Urinary μg/24 hr.	179	185	141	142	101	151
Faecal μg/24 hr.	259	778	647	614	570
Fasting μg/hour	12	9	13	12	8	11
Test dose % return	18.4	10.5	9.7	11.5	8.8	11.6

TABLE 3 — (continued)

SUBJECT HEIGHT, CM WEIGHT, KG	1 165 46.3	2 169 71.7	3 161 45.8	4 170 56.2	5 160 56.2	6 152 50.8	7 164 58.5	8 175 62.6	9 165 59.0	10 158 59.4	11 174 63.5	12 170 69.4	AVERAGE
Period III													
Intake													
Total Mg/1000 Cal.	1360 0.65	1134 0.64	1244 0.65			1347 0.63				1253 0.68		1063 0.72	1234 0.66
Excretion													
Urinary μg/24 hr.	345	214	234			287				229		269	263
Fecal μg/24 hr.	415	656	570			657						574	574
Fasting μg/hour	8	11	15			14				8		9	11
Test dose % return	26.5	17.9	12.9			13.9				11.1		15.5	14.3
Period IV													
Intake, total													
Week 1	7252	7071	7210			7213				7169		7066	7164
Week 2	7257	7045	7232			7234				7210		7065	7177
Excretion, urinary/24 hr.													
Week 1	4275	4835	3968			4254				3261		2917	3935
Week 2	4847	5805	4958			4538				4547		3823	4753
Period V													
Intake													
Total Mg/1000 Cal.	1291 0.59	1086 0.54	1254 0.68			1275 0.59				1267 0.59		1067 0.76	1206 0.63
Excretion													
Urinary μg/24 hr.	403	504	299			243				212		286	325
Fecal μg/24 hr.	361	856	611			628						540	599
Fasting μg/hour	16	15	14			10				16		11	13
Test dose % return	18.1	17.0	10.2			24.6				14.6		9.1	15.6

¹ Daily intakes and urinary excretions during periods I-a, I-b, II, and III represent the average of 3-day collections made weekly throughout these periods, with the exception of those for subject 3 in periods I-a and I-b which represent a single 3-day collection at the end of these periods.

² This figure is not included in the average. Due to an error the 4-hour collection was not made following the test dose, and a second test dose was given the following day. This is the return of the second test dose.

daily urinary excretion was almost exactly the same as it had been during period III for five of the six subjects. Here again subject 2 was the exception, her daily excretion being almost double that observed in period III. It should be pointed out that the results shown for period V represent only one 3-day collection. Throughout the course of the experiment there were a few cases in which the average daily excretion for a particular week did not fall exactly in line with the results secured during the rest of the period. It is possible that something of this nature may have occurred in the case of this particular collection. In view of the other results secured on subject 2, however, it seems probable that she did have more riboflavin "available" for excretion than did the other subjects.

Fasting 1-hour excretion

During periods I-a and I-b, when the average intake was 0.29 mg/1000 Cal., the fasting 1-hour excretion dropped sharply, and at the end of period I-b it averaged 6 μ g. When the riboflavin intake was increased to an average of 0.49 mg/1000 Cal., the fasting excretion rose to an average of 11 μ g per hour. There was no further increase in the fasting excretion when the intake was increased to 0.66 mg/1000 Cal. in period II, nor was the excretion in period V (13 μ g per hour) following the supplementation in period IV, appreciably higher than in period III.

The fasting 1-hour excretions were also calculated on a unit volume basis as suggested by Feder, Lewis and Alden ('44). It was found that the values for excretion per hour were more constant than the values for excretion per unit volume.

Test dose returns

A sharp drop in test dose returns was found to have occurred at the end of 59 days on an average intake of 0.29 mg/1000 Cal. At that time four of the nine subjects excreted 1% or less of the test dose, while four others returned between 2 and 4%. One person, subject 2 again, showed a much higher

return than anyone else in the group — 11.6%. At the end of period I-b the average return of a test dose was slightly higher, despite the fact that there had been no increase in the amount of riboflavin in the diet. If subject 1, who showed an unusually large increase in test dose return (from 0.3 to 7.0%) is not included, the average returns are 3.1 and 4.2% for periods I-a and I-b, respectively, which still indicate a slight increase. Although the level of riboflavin in the diet was not changed at the end of period I-a, the thiamine was increased from an average of 0.14 to 0.20 mg/1000 Cal. It is possible that this increase in thiamine may have had some effect on the utilization of riboflavin.

At the end of period II there was a sharp increase in the amount of the test dose returned, the average at this time being 11.8%. There was a further increase, to an average of 14.3%, at the end of period III. Apparently no additional benefit was derived from the 2-week period of vitamin supplementation; the average amount returned at the end of period V was 15.6%, practically the same as the average return at the end of period III. Two of the six subjects showed an increased return; the other four returned approximately the same amount or slightly less than in period III. According to the theory of Melnick ('42) this would indicate that the tissue stores of riboflavin had been satisfactorily filled on the 0.66 mg/1000 Cal. level of intake, since the 2-week period of high intake did not affect the test dose return.

Fecal excretion

Although there was considerable variation in the total amount of riboflavin excreted in the feces by different individuals, the level for each person remained relatively constant. Apparently the fecal excretion was not influenced either by changes in the dietary level of riboflavin, or by the fact that the starch content of the diet was higher during the early periods of the study, when larger amounts of cereal foods were necessary to maintain the caloric intake.

That intestinal synthesis of riboflavin did occur is shown by the fact that in some cases the fecal excretion alone was higher than the dietary intake of the vitamin. In a number of other cases the total urinary and fecal excretion was higher than the intake. Whether any absorption or utilization of this synthesized vitamin took place is difficult to determine.

General health

No definite signs or symptoms of riboflavin deficiency were observed at the time of the original examination, or in the examination at the end of period I-a. No cheilosis was observed among any of the subjects at any time, nor did any seborrheic dermatitis occur. The eyes were not examined with a slit lamp, but a magnifying glass failed to reveal any abnormal vascularization of the cornea. During the first examination the tongue of one of the young women (subject 3) was observed to show slight enlargement and reddening of the fungiform papillae, and some indentation of the margin. This condition did not change throughout the study. When the special examination of the mouth, tongue, and eyes was made at the end of period I-b, two other subjects were observed to have a similar condition of the papillae, and one showed slight indentations of the margin of the tongue. Two subjects were found to have slight photophobia when the eyes were exposed to a strong light. One of these was subject 11, whose daily excretion and return of a test dose were low. The other was subject 2, whose excretions were high. All of the changes were slight, however, and the clinician who made the examination stated that they were "not convincing and of doubtful significance."

Constipation was observed by certain of the subjects during periods I-a and I-b, and was severe in several cases. This occurred in spite of the fact that fruit juice, raw or cooked fruit, and a cooked and a raw vegetable were included in the meals each day. The condition was relieved immediately when the diet was changed in period II, at which time both the riboflavin and thiamine contents of the diet were increased.

At the end of period I-b four young women withdrew from the study because of unfavorable subjective symptoms. All had severe constipation and all complained of fatigue, lack of concentration, desire to sleep, and general malaise. It seems likely, however, that these symptoms, if caused by the diet, were due to a low intake of thiamine rather than of riboflavin.

Interpretation of results

A graphic comparison of the average intake and excretion of riboflavin by all of the subjects throughout the course of the study is found in figure 1. From the pronounced drop in

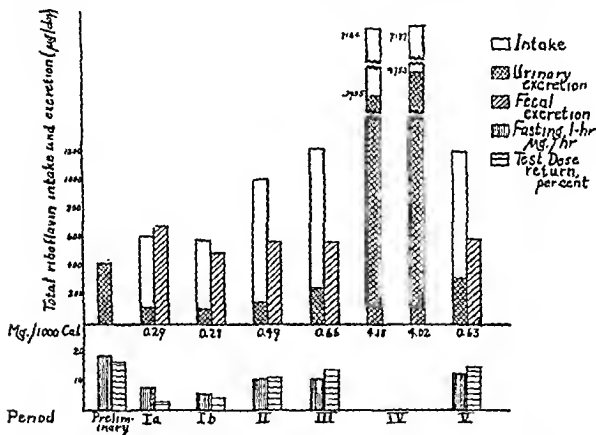


Fig. 1 Average intake and excretion of riboflavin during each experimental period.

daily excretion, fasting excretion, and test dose returns which occurred during period I, when the riboflavin content of the diet was restricted to 0.29 mg/1000 Cal., it seems evident that the riboflavin in the body tissues reached a low level during this period.

Although the 24-hour excretion did not increase greatly when the riboflavin intake was raised to an average of 0.49 mg/1000 Cal., there was a striking increase in both the fasting excretion and the amount of a test dose returned. The data on the test dose returns indicate that the concentration of tissue riboflavin had been increased during this period.

The rise in 24-hour excretion in period III (on an average intake of 0.66 mg/1000 Cal.) was greater in proportion than might have been expected from the dietary increase in riboflavin. The average increase in riboflavin intake in each period, together with the resulting increase in excretion are shown in table 4. The percentage excreted, both of the total intake and of the added dietary riboflavin in each period is also shown in table 4.

TABLE 4

Percentage of the total intake and of the increase in intake which was excreted at each dietary level.

PERIOD	TOTAL INTAKE	INCREASE IN INTAKE	TOTAL EXCRETION	INCREASE IN EXCRETION	TOTAL INTAKE EXCRETED	INCREASE IN INTAKE EXCRETED
	μg	μg	μg	μg	%	%
I	600		113		19	
II	1017	417	150	37	15	9
III	1234	217	263	113	21	52
IV	7171	5937	4344	4081	61	69
V	1206		325		27	
V ¹	1230		289		23	

¹ Average if subject 2 is not included.

There was little variation in the average percentage of the total intake excreted, except during the period of supplementation. The figures on the percentage of total intake excreted probably are not so significant, however, as those on the percentage of the added riboflavin which was excreted in each period. When the riboflavin intake was increased from an average of 0.29 to 0.49 mg/1000 Cal. in period II (a total increase of about 400 μg per day) nearly all of the added riboflavin was "retained" by the body, only 9% of the added

amount being excreted. When 200 μ g per day were added in period III to make an intake of 0.66 mg/1000 Cal., more than 50% of the added amount was excreted. It seems logical to assume that when a relatively small increase in riboflavin intake results in an excretion of more than 50% of that increase, the added amount was not necessary.

The fact that both the daily urinary excretion and the test dose returns were increased when the average intake was 0.66 mg/1000 Cal. in period III, together with the fact that there was no apparent benefit from a 2-week period of vitamin supplementation, would indicate that this level of riboflavin intake probably is more than enough to take care of bodily needs for the vitamin. The observation that despite the low daily excretion of riboflavin, the fasting 1-hour excretion and the return of a test dose rose on an intake of 0.49 mg/1000 Cal. suggests that this level of intake supplies at least the minimal "requirements." This seems even more evident when it is considered that this level of intake followed a long period of low intake during which there was evidence that the tissue concentration of riboflavin had been decreased. This amount of riboflavin is the same as that found by Williams and his co-workers ('43) to supply the minimal requirements of riboflavin for women. This evidence, together with the data presented herewith which indicate that an intake of 0.66 mg/1000 Cal. supplied more of the vitamin than was necessary, strongly suggests that the riboflavin "needs" of healthy, active young women can be met by an intake of about 0.50 mg/1000 Cal., or a total intake of approximately 1 mg.

SUMMARY

The riboflavin excretion of young women on a diet in which the amount of the vitamin was progressively increased was followed during an 8-month period.

From an initial intake during periods I-a and I-b of 0.29 and 0.28 mg/1000 Cal., the riboflavin level was increased by

the addition of milk and by other dietary changes to 0.49 and 0.66 mg/1000 Cal. during periods II and III. In period IV, which lasted 2 weeks, the total daily intake was 7.1 mg. During period V, 3 weeks long, the intake averaged 0.63 mg/1000 Cal.

Physical examinations at the beginning of the experiment and following periods I-a and I-b revealed no signs of riboflavin deficiency.

Daily urinary excretions "levelled off" within 10 days after each change in diet. Average daily excretions during the first four periods were 119, 107, 150, and 263 μ g, respectively. Excretions rose sharply during supplementation, but after 2 weeks on a lower intake the average excretion was 325 μ g.

Levels of fecal excretion of riboflavin differed considerably among individuals, but the amount excreted by each person remained relatively constant despite dietary variations.

Test dose returns at the end of periods I-a and I-b averaged 2.8 and 4.5%. With increased intake in periods II and III, the average returns were raised to 11.8% and 14.3%, respectively. Test dose returns were not increased by supplementation, the average at the end of period V being 15.6%.

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THIAMINE EXCRETIONS AND BLOOD LEVELS OF YOUNG WOMEN ON DIETS CONTAINING VARY- ING LEVELS OF THE B VITAMINS, WITH SOME OBSERVATIONS ON NIACIN AND PANTOTHENIC ACID¹

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ONE FIGURE

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Studies of the thiamine requirement of women based on the presence or absence of deficiency symptoms have been made by Elsom ('42), and Williams ('40, '42, '43a, '43b) and their associates. Elsom and her co-workers ('42) reported that six subjects who received a daily intake of less than 600 μ g all developed clinical signs of deficiency and had daily urinary excretions of 42 μ g or less. Three other subjects maintained on 650 to 775 μ g per day showed no signs of deficiency for approximately a month and had urinary excretions of 46 to 60 μ g. They concluded that the minimal thiamine intake compatible with health was about 650 μ g and the thiamine-calorie ratio, about 0.35. Williams and his associates ('40, '42, '43b), who have made an extensive series of studies, have repeatedly reported definite signs of clinical deficiency, low urinary excretions and low test dose returns when women were maintained on daily intakes of less than 0.40 mg per 1000 cal. In an additional long-time experiment on five subjects who were receiving intakes of 0.45 mg per 1000 cal. these authors ('43a)

¹The expenses of this study were defrayed in part by funds granted by the National Dairy Council on behalf of the American Dairy Association and by the Williams-Waterman Fund of the Research Corporation.

found some increase in blood pyruvate following glucose administration in all but one subject and depletion of tissue stores as evidenced by daily urinary excretions of less than 100 μ g and test dose returns of less than 20% in some subjects. They have concluded that 0.45 mg of thiamine per 1000 cal. cannot be regarded as representing more than the minimal requirement.

Melnick ('42) analyzed the daily urinary excretions and test dose returns of a large number of individuals, both men and women, on different thiamine intakes. He concluded that a daily intake of 1 mg or 0.35 mg per 1000 Cal. resulted in excretions indicative of adequate tissue stores. However, since he believed that a margin of safety was desirable, he recommended an intake of 0.50 mg per 1000 cal.

Approximately the same value was obtained by Foltz, Barborka and Ivy ('44) as the result of a study of young men where both work output and urinary excretions were used as criteria. In the series of experiments conducted by Keys and his associates ('43, '45), however, no evidences of physical impairment and no decrease in work output was observed when young men were maintained for several months on intakes of 0.23 mg per 1000 Cal., although the urinary excretions and test dose returns were low. On a still lower intake of 0.185 mg per 1000 Cal. these authors found no signs of deficiency except an elevation in the resting level of blood pyruvate and a slight but similar tendency after work. The daily urinary excretions on this level of intake averaged only 7 μ g during the last 3 weeks of the study. It is worthy of note, however, that the total thiamine intakes of Keys' men, because of their higher caloric intakes, were much higher than those of the smaller, less active women subjects of Williams and Elsom.

The present study, which was carried out in conjunction with one on riboflavin reported by Davis, Oldham and Roberts ('46), was undertaken in order to secure additional information on the amount of thiamine necessary to maintain reasonable tissue concentrations of this vitamin in young women

as evidenced by thiamine excretions and blood levels. That changes in the tissue concentration of thiamine are reflected by such measurements has been demonstrated by Hulse and co-workers ('44). These authors found definite decreases in the yeast stimulating activity of skeletal muscle and blood and in the urinary excretion of thiamine of six young men on a low thiamine diet as compared to that on their self-selected diets.

The intakes and excretions of niacin and pantothenic acid were also determined throughout the study, although at less frequent intervals than those of thiamine.

METHODS OF STUDY

This study was carried out in conjunction with the one on riboflavin. The general plan and the subjects have been described elsewhere by Davis et al. ('46). Therefore, only a brief review will be given here.

After a preliminary period of 7 days in which the urinary excretions of thiamine, the blood levels and the returns of test doses were determined on a freely chosen diet, the experiment ran continuously for 226 days. This was divided into four main periods which varied in length from 41 to 59 days. They were followed by a 14-day period of supplementation after which there was a 20-day period on intakes which were approximately the same as those previous to the supplementation. Diets were given which were adequate except for their riboflavin and thiamine contents. The intakes of these vitamins were low in the first period and were gradually increased as the study progressed. The thiamine levels amounted to 0.14, 0.20, 0.36 and 0.51 mg per 1000 Cal. and the riboflavin levels to 0.29, 0.28, 0.49 and 0.66 mg per 1000 Cal. in the first four periods. Daily supplements of 6 mg of riboflavin, 4 mg of thiamine, 10 mg of niacin and 0.5 mg of pantothenic acid were added in the next period and in the final period the intakes of thiamine and riboflavin were 0.54 and 0.63 mg per 1000 Cal., respectively.

Seventy-two-hour food composites and urine collections were made weekly. At the end of each period one or more

1-hour fasting urine collections and two 4-hour urine collections, one before and one after the administration of a test dose of thiamine, were made. Samples of blood were also taken at the end of each period.

The kinds and amounts of foods served, their preparation, the supplements of nutrients other than the B vitamins, the adequacy of the diet, the method used in making composites of individual intakes for analyses and the collection and treatment of urinary and fecal excretions have been reported by Davis et al. ('46). The administration of the oral test dose of 20 μ g of thiamine² per kg of body weight was similar to that of the riboflavin test dose but was always given the day after the riboflavin test dose in order that the excretion of riboflavin would not be affected (Klopp, Abels and Rhoads, '43). The method of calculation of the test dose return wherein a correction was made for the basal excretion by subtracting a 4-hour control sample, was the same as that used in the case of riboflavin.

The food composites, fecal collections, and all urine samples except the fasting 1-hour specimens in certain periods were analyzed for their thiamine content by the method of Hennessy and Cerecedo ('39) as modified by Najjar and Ketron ('44). The fasting 1-hour specimens in all but the last two periods were analyzed by the microfermentation method of Atkin, Schultz and Frey ('39), as described by Knott, Kleiger and Torres-Bracamonte ('43), because of their extremely low thiamine content. The blood samples were also analyzed by the latter method.

The 72-hour food composites representing the last week of each period, except the one in which the supplements were given, and the corresponding urinary and fecal collections were assayed for nicotinic acid by the microbiological method of Krehl, Strong and Elvehjem ('43) and for pantothenic acid³ by the method of Neal and Strong ('43) with certain

²Thiamine was generously supplied by Merck and Company.

³During the periods in which pantothenic acid was determined, separate aliquots of urine collections and food composites were prepared by autoclaving without the addition of acid and preserving with toluene.

TABLE 1 (continued)

Urinary thiamine excretions and blood levels on different intakes.¹

SUBJECT	1	2	3	4	5	6	7	8	9	10	11	12	AVERAGE
HEIGHT, CM	165	169	161	170	160	152	164	175	163	158	174	170	
WEIGHT, KG	46.3	71.7	45.8	56.2	56.2	50.8	58.5	62.6	59.0	59.4	63.5	69.4	
Period III (41 days)													
Intake:													
μg/24 hr.	962	883	952			979				949		890	937
Mg/1000 Cal.	0.47	0.55	0.49			0.49				0.52		0.54	0.51
μg/kg	20.8	12.3	20.8			19.3				16.0		12.9	17.0
Excretion:													
Urinary													
μg/24 hr.	139	80	77			144				135		68	107
Fasting													
μg/hour	9	12	5			7				3		2	6
Test dose													
% return	10.4	5.2	6.1			8.1				10.0		4.0	7.3
Blood level													
μg/100 ml	5.9	5.6	5.4			6.4				5.4		4.8	5.6
Period IV (14 days)													
Week I													
Intake:													
μg/24 hr.	4964	4862	4938			4919				4896		4822	4900
Excretion:													
Urinary													
μg/24 hr.	856	707	903			792				939		641	805
Week II													
Intake:													
μg/24 hr.	4968	4849	4951			4941				4917		4856	4914
Excretion:													
Urinary													
μg/24 hr.	1458	1477	1388			1592				1522		1199	1439
Period V (20 days)													
Intake:													
μg/24 hr.	1087	972	1064			1054				1050		936	1027
Mg/1000 Cal.	0.50	0.49	0.57			0.49				0.49		0.67	0.54
μg/kg	23.5	13.6	23.2			20.7				17.7		13.5	18.7
Excretion:													
Urinary													
μg/24 hr.	254	214	147			204				212		146	196
Fasting													
μg/hour	16	13	9			7				6		7	10
Test dose													
% return	10.6	9.1	10.8			13.2				14.3		6.9	10.8
Blood level													
μg/100 ml	6.3	5.5	6.1			6.2				6.7		6.3	6.2

¹ Daily intakes and urinary excretions during periods I-a, I-b, II and III represent the average of 3-day collections made weekly throughout these periods with the exception of those for subject 3 in periods I-a and I-b which represent a single 3-day collection at the end of these periods.

² Values for the three subjects who did not participate in the first three periods are not included in this average.

stipation. At the end of the period, however, another careful examination was made and again no deficiency symptoms were observed.

The thiamine intake was increased to approximately 400 μg or 0.20 mg per 1000 Cal. in period 1-b. The daily urinary excretions increased only slightly but the blood levels and the fasting 1-hour excretions returned to approximately the same values as were found in the preliminary period. In contrast, the test dose returns were even lower than in period 1-a. They averaged only 0.7% with only two subjects returning more than 0.8%. At this time, although no deficiency symptoms were apparent, the complaints of the same four subjects (7, 8, 9 and 11) increased. In spite of the greater thiamine intake, their constipation had become more severe; in addition they complained of "difficulty in grasping ideas" and "inability to concentrate." Because they felt that their scholastic standings were being jeopardized, these four subjects withdrew from the experiment at this time. Of the five remaining, one (3) was in a highly nervous state and also found it difficult to concentrate, but continued on the experiment. The others, when questioned, reported that they had noticed no change in their state of health.

Although these symptoms are admittedly subjective, the facts (1) that these subjects were the ones who had the poorest tissue stores at the beginning of the study; (2) that their symptoms were the same as some of those reported by Elsom ('42), Williams ('40, '42, '43b), Foltz ('44) and their co-workers, and (3) that subject 3 who remained on the experiment, improved markedly on the next level of intake, lend credence to the belief that they were manifestations of a subclinical thiamine deficiency.

In period II the average daily thiamine intake was increased to slightly over 700 μg or 0.36 mg per 1000 Cal. The daily excretions showed a small increase but still averaged less than 50 μg . The fasting 1-hour excretions increased slightly and the blood levels of all rose to 6 μg or more per 100 ml. The test dose returns, however, remained low. The average re-

turn for the group was only 4.0% and only one subject (1) returned more than 5.0%. It would seem that, as judged by the usual standards, none of the intakes up to this point was adequate for this group of young women.

In period III when the intake was increased to approximately 900 μg or 0.51 mg per 1000 Cal., the daily urinary excretions averaged 107 μg , although those of three of the six subjects still were only 80 μg or less. A comparison of the average fasting 1-hour excretions, blood values and test dose returns of this period with those of period II may not be justifiable because three new subjects entered in period III. If the results of the subjects who participated in both periods II and III (subjects 1, 2 and 3) are considered, it will be seen that the fasting 1-hour excretions showed no definite trend, that the blood values were lower in every instance,^a but that the test dose returns were all higher. The return of only one subject (1), however, was above 10% and of the three new subjects, only one had a test dose return comparable to that which she had on a freely chosen diet. These considerations would suggest that the thiamine concentration of the tissues of these subjects had increased only moderately on this level of intake; that the tissues were by no means "saturated."

That this was the case is also borne out by the results obtained in period V after 2 weeks of supplementation in period IV. The average intake in period V was 0.54 mg per 1000 Cal., approximately the same as in period III. At the end of this period the daily excretions of all the subjects were higher than they were previous to the supplementation; the fasting 1-hour excretions were higher in five and the blood levels were higher in four of the six subjects and the test dose returns were larger in all but one case (1), the average return for the group being 10.8%. According to the concept of Melnick, Field and Robinson ('39), if the tissues of these subjects had been saturated with thiamine in period III, the 2 weeks'

^aThe lower blood levels cannot be explained unless those at the end of period II were unduly high because of atmospheric conditions existing at that time which might have had a dehydrating effect.

supplementation in period IV would not have caused increased excretions at the end of period V, 2 weeks after the supplementation had been discontinued. In all but one case (subject 1) these subjects did show definitely increased excretions. It would seem, therefore, that the tissue concentrations of only one of these subjects (1) approached saturation on the highest level of intake studied. The fact that the daily urinary excretions of all the subjects were greatly increased in the last 3

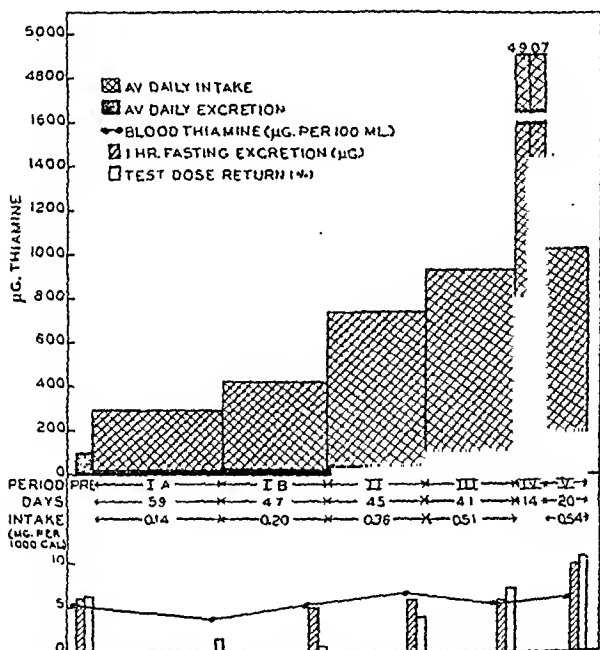


Fig. 1 Average daily excretions of thiamine, test dose returns, fasting excretions and blood thiamine at different levels of intake.

days of supplementation as compared to the first 3 days gives further support to this view and suggests that saturation had not been reached on the 0.51 mg per 1000 Cal. level even in the case of subject 1.

In interpreting these results in terms of practical allowances, it is recognized that a higher "requirement" is usually arrived at when the intakes are being progressively in-

creased from a state of depletion, than when they are being decreased from higher levels. It is possible, then, that this level of 0.5 mg per 1000 Cal. might have been sufficient to maintain tissue concentrations after they had once been increased. It should be noted, however, that although the thiamine-calorie ratios were of the order generally considered to be satisfactory, the calorie intakes and hence the total thiamine intakes were relatively low. All were less than 1 mg and two were less than 0.9 mg. For two of the largest subjects (2 and 12) whose test dose returns were only 5 and 4%, this provided only 12-13 μ g per kg; for the others whose returns were somewhat higher, it amounted to 16 to 21 μ g per kg. It is possible that the thiamine-calorie ratio alone is not a satisfactory basis for predicting thiamine needs, especially at low calorie intakes. These results suggest that the total daily thiamine intake of young women should probably not be less than 1 mg or 20 μ g per kg of body weight regardless of the actual calories consumed. At a 2000-cal. level this would provide a thiamine-calorie ratio of 0.50. This is approximately the ratio suggested as adequate by Melnick ('42), Williams and associates ('43a), and Foltz, Barborka and Ivy ('44).

Fecal excretions of thiamine

The fecal excretions of total thiamine⁷ which were determined for some of the subjects during the last week on the different levels of intake (table 2) showed no consistent relation to the intakes. Only one subject (3) showed a gradual increase in excretion as the intakes were increased and it was not always large enough to be considered significant. It will be noted, however, that three of the four subjects did have significantly higher excretions in period II when the daily intake was increased to approximately 700 μ g than in period 1-b when the daily intake was in the neighborhood of 400 μ g.

⁷ The fecal analyses were made by Lois Buttles. The results were reported in detail in her master's thesis, "The Total Thiamine Excreted Through the Gastrointestinal Tract by Human Subjects." University of Chicago, 1945.

TABLE 2

Average daily urinary and fecal thiamine excretions on different intakes.¹

SUBJECT	1	2	3	4	5	6	7	12
Period I-a								
Intake, μg	288	303	305	...	343	...
Excretion:								
Urine, μg	22	25	24	...	22	...
Feces, μg	267	108	351	...	211	...
Total, μg	289	133	375	...	233	...
Period I-b								
Intake, μg	366	375	402	392	372
Excretion:								
Urine, μg	29	16	19	29	10
Feces, μg	405	213	306	104	221
Total, μg	434	229	325	133	231
Period II								
Intake, μg	710	640	741	738
Excretion:								
Urine, μg	78	65	41	35
Feces, μg	459	354	318	221
Total, μg	537	419	359	256
Period III								
Intake, μg	1041	974	1046	1063	...	1010
Excretion:								
Urine, μg	116	107	97	160	...	68
Feces, μg	348	323	366	35	...	41
Total, μg	464	430	463	195	...	109
Period V								
Intake, μg	1087	972	1064	1054	...	936
Excretion:								
Urine, μg	254	214	147	204	..	146
Feces, μg	437	430	388	65	..	31
Total, μg	691	644	535	269	..	177

¹ All figures in this table are based on collections made for 3 days during the last week of each period.

There was little direct evidence of bacterial synthesis of thiamine by the intestinal bacteria as only two subjects actually excreted more thiamine than they received in their diets and this occurred in only one period in both cases. There are certain indications, however, that it did occur. The subjects who began on the low levels of thiamine and riboflavin intake (1, 2 and 3) excreted from 7 to 10 times the amount of thiamine in periods III and V as subjects 6 and 12 who entered the experiment later and had never been on the low levels. Since they were all receiving the same diet in the later periods, either some factor related to their earlier diets must have caused the increased excretions or there were pronounced individual differences. It is unfortunate that fecal collections were not made in the preliminary period, for if something in the experiment was the cause of the increased output, the effect must have taken place in period I-a, since all the subjects excreted higher amounts of fecal thiamine in that period than subjects 6 and 12 did in periods III and V.

There were many differences in the diets of period I-a as compared to periods III and V, which may have exerted an influence on the intestinal flora. The amounts of both riboflavin and thiamine were much lower in period I-a; there were more cereals and less vegetables and fruit in period I-a; the subjects consumed much larger amounts of bread in period I-a and subjects 1, 2 and 3 received, on the average, 5.8% more carbohydrate in period I-a than in periods III and V. Which, if any, of these factors operated to cause an increased synthesis of thiamine, it is difficult to say.

Niacin and pantothenic acid

Both intakes and excretions were analyzed for niacin and pantothenic acid the last week of each period with the exception of period IV, when all collections were analyzed. Although the findings were not conclusive, they are presented here because of the paucity of data concerning the intakes and excretions of these vitamins. The individual results and the averages for the group are presented in table 3.

The average daily niacin intakes of the subjects ranged from 8.7 to 14.5 mg previous to supplementation, and were 23.4 mg during this period. In spite of this wide range in intake, the average urinary excretions remained relatively constant in the neighborhood of 1.0 mg. This represented approximately 10% of the lower and 5% of the higher levels of intake. The urinary excretion of approximately 1.0 mg is in agreement with the findings of Sarett, Huff and Perlzweig ('42) and Goldsmith ('42, '44), on normal individuals who were consuming different types of diets. Briggs and co-workers ('45) also have reported continued urinary excretions of approximately this magnitude by a subject whose daily niacin intake was 3 mg or less for a period of 42 weeks. This tendency toward constant niacin excretions is unlike that of both riboflavin and thiamine, which respond promptly to changes in intake.

The average fecal excretions of niacin on the lowest levels of intake in periods I-a and I-b were approximately the same as the urinary excretions but were somewhat higher on the higher intakes. However, they were never more than 15% of the intake. For the most part both the urinary and fecal excretions of the different subjects did not vary greatly from the average.

Both the intakes and excretions of pantothenic acid found in this study were of the same order of magnitude as those found by other workers on individuals consuming self chosen diets. The low levels of intake of these subjects correspond closely to the average intake of 2.4 mg found in a group of twenty-four women with low incomes; the higher levels to that of 4.8 mg in a group of twelve women with moderate incomes (Winters and Leslie, '43, '44). The urinary excretions on the higher levels of intake approximated the average amounts of 3.8, 3.2 and 3.4 mg reported on groups of normal individuals by Pelczar and Porter ('41), Pearson ('41) and Wright and Wright ('42).

The pantothenic acid excretions present a decidedly different picture from those of niacin. At every level of intake

from 2.1 to 4.7 mg, the average total excretions consistently approximated the intakes (table 3). When individual values are considered, the tendency was for the excretions to exceed the intakes by small amounts. From 54 to 84% of the excreted pantothenic acid was found in the urine and from 25 to 38% in the feces. This agrees with the findings of Gardner, Neal, Peterson and Parsons ('43) although their subjects excreted a slightly higher proportion in the urine and a smaller one in the feces.

These results are also in harmony with the suggestion of the Wisconsin group that either no pantothenic acid is destroyed in the body or that intestinal synthesis compensates for the destruction in the tissues. That synthesis could adjust to different intakes as precisely as was the case in this study, however, seems rather doubtful. In view of the findings here presented the former theory seems more plausible. In any case, the validity of a daily allowance of the 10 mg suggested by Williams ('42) is questionable.

SUMMARY

Daily urinary and fecal thiamine excretions, test dose returns, blood thiamines and 1-hour fasting thiamine excretions of twelve women were determined on various levels of intake over a period of 8 months. Intakes of 0.14, 0.20 and 0.36 mg per 1000 Cal. were judged to be inadequate on the basis of urinary excretions and test dose returns. On an intake of 0.51 mg per 1000 Cal. the excretions increased somewhat but still remained low. That the tissues were not saturated at this level was further indicated by the facts that (1) during subsequent supplementation the excretions were higher the last 3 days than the first 3; and (2) in the final period on an intake similar to that previous to supplementation, both daily urinary excretions and test dose returns showed increases. It is suggested that the total daily thiamine intake should probably not be less than 1 mg or 20 μ g per kg of body weight.

Fecal thiamine excretions for different individuals were relatively constant throughout the study. Average daily uri-

mary and fecal excretions of niacin also remained relatively constant at approximately 1.0 mg each, regardless of the levels of intake. Total excretions of pantothenic acid closely approximated the intakes at each level, by far the larger proportion being excreted in the urine.

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THE ISOLEUCINE, LEUCINE AND VALINE REQUIREMENTS OF THE CHICK

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The qualitative requirements of the chick for leucine, isoleucine and valine have been reported previously (Grau and Almquist, '44; Hegsted, '44; Almquist and Grau, '44). In these studies it was found that the omission of any of these amino acids from the diet caused an immediate loss in weight and eventual death. The present report is concerned with the effects on growth of different levels of these amino acids in the diet and with the effectiveness of their optical isomers in promoting growth.

METHODS

White Leghorn chicks were fed a commercial-type mash until they were 10 days old. From the tenth to the fourteenth day they were placed on a conditioning diet with the following composition (in grams): cellulose¹ 5, calcium gluconate 8, mineral mixture 3.8, crude soybean oil 5, sardine oil (400 D-3000 A per gm) 0.25, natural mixed tocopherols² 0.05, choline chloride³ 0.2, inositol 0.1, cholic acid 0.1, 2-methyl-1, 4-naphthohydroquinone diacetate 0.001, thiamine 0.001, riboflavin 0.001, pyridoxine 0.001, nicotinic acid 0.003, calcium (d) panto-

¹ Cellu flour.

² Natural mixed tocopherols (15%), Distillation Products, Inc.

³ Choline chloride was provided by Lederle Laboratories, Inc., through the courtesy of Dr. T. H. Jukes.

thenate 0.003, biotin ⁴ 0.00001, folic acid ⁵ 0.0001, casein 25, gelatin 7, and glucose ⁶ to make 100 gm. The mineral mixture contributed the following materials in grams to each 100 gm of diet: tricalcium phosphate 2.0, dipotassium phosphate 0.5, potassium chloride 0.3, manganese 0.01, silicon 0.046, magnesium 0.048, aluminum 0.008, iron 0.014, copper 0.001, zinc 0.001, iodine, 0.0008, and cobalt 0.0005 gm.

The chicks were weighed daily until they were 2 weeks old, when groups of four closely selected chicks were fed the experimental diets. The selection was made so that all groups had approximately equal weight distributions and average weights. Only chicks which grew uniformly during the second week were used. The range of weights of the chicks chosen was between 84 and 100 gm for the first experiment and between 72 and 99 gm for the second. The chicks were housed in electrically heated battery brooders with wire floors. Feed and water were supplied ad libitum. The basal experimental diet was the same as the conditioning diet except for the amino acid source, which in the experimental diet consisted of a mixture of commercial amino acids. The amino acid mixtures used as positive controls in the two experiments are shown in table 1. Sodium bicarbonate was added to neutralize the hydrochlorides of the basic amino acids and to provide sodium chloride, which was omitted from the mineral mixture used. As the amount of an amino acid in the diet was decreased, an equal weight of glucose was added to make up the difference.

The amino acids used were commercial products, except for the optical isomers of isoleucine and valine, which were prepared according to the methods of Locquin ('07) and Fischer ('06), respectively. The optical rotations of these preparations are given in table 2.

⁴ Biotin was furnished by Merck and Co., Inc., through the courtesy of Dr. J. C. Keresteszy.

⁵ Folic acid was provided by Lederle Laboratories, Inc., through the courtesy of Dr. E. L. R. Stokstad.

⁶ Cerelose.

TABLE 1

Mixtures of amino acids used in the positive control diets.

AMINO ACID	FORM	LEVEL AS FED		AMINO ACID	FORM	LEVEL AS FED	
		Expt. 1	Expt. 2			Expt. 1	Expt. 2
		%	%			%	%
Alanine	dl	1.5	1.5	Lysine	1(+)-HCl·H ₂ O	1.4	1.4
Arginine	1(+)-HCl	1.4	1.4	Methionine	dl	0.6	0.6
Aspartic acid	dl	1.0	..	Norleucine	dl	0.2	..
Cystine	1(-)	0.5	0.5	Phenylalanine	dl	1.5	1.0
Glutamic acid	1(+)	5.0	7.0	Proline	1(-)	2.0	..
Glycine	1.8	1.8	Threonine	dl	3.0	1.3
Histidino	1(+)-HCl·H ₂ O	0.8	0.8	Tryptophane	1(-)	0.3	..
Hydroxyproline	1(-)	0.2	..	Tryptophane	dl	..	0.6
Isoleucine	dl	2.0	1.0	Tyrosine	1(-)	2.0	2.0
Leucine	1(-)	2.0	1.5	Valine	dl	2.0	1.5
				(NaHCO ₃)		1.5	1.5

TABLE 2

Specific rotations of the isomers of isoleucine and valine in 20% HCl at 20°C.

AMINO ACID	FOUND	RECORDED IN REFERENCE	REFERENCE
l(+)-Isoleucine	+ 39.1°	+ 40.61	Locquin ('07)
d(-)-Isoleucine	- 39.0°	- 40.86	Locquin ('07)
l(+)-Valine	+ 28.6°	+ 28.7	Fischer ('06)
d(-)-Valine	- 28.2°	- 28.4	Fischer ('06)

The chicks and feed were weighed daily during the experiments, which lasted 10 days. Individual growth curves were plotted, and from these a smooth curve was drawn for each chick. The initial and final corrected weights were obtained from the curve, and the per cent gain per day was calculated according to the formula:

$$\% \text{ gain per day} = \frac{\text{ave. gain} \times 100}{\text{ave. wt.} \times \text{no. of days}}$$

In order to obtain the ratio of gain to feed, the sum of the corrected weight gains was divided by the total feed consumed.

RESULTS AND DISCUSSION

The growth rates with the various diets as well as the range of the individual rates are given in table 3.

TABLE 3

The effects of different levels of isoleucine, leucine and valine on the rate and efficiency of gain. There were four chicks in each group; the experiment lasted 10 days.

AMINO ACID	LEVEL OF AMINO ACID IN DIET	OPTICAL FORM	PER CENT GAIN PER DAY		GAIN PER GM FEED CONSUMED
			mean	range	
Isoleucine	%				gm
	0	..	-1.3	-1.9, -0.5	-0.23
		d(-)	-0.7	-1.9, +0.3	-0.12
	0.45 ¹	dl	+1.3	+0.1, 2.0	+0.15
		l(+)	4.1	3.3, 5.2	0.35
	0.5	dl	1.2	0.0, 2.1	0.17
	1.0	dl	4.0	3.3, 4.3	0.42
	1.5	dl	3.6	3.1, 4.0	0.40
	2.0	dl	3.3	2.2, 4.7	0.39
Leucine	0	..	-2.7	-3.2, -1.6	-0.53
	0.5	l(-)	-1.1	-1.4, -1.0	-0.24
	1.0	l(-)	+1.8	+1.5, 2.2	+0.24
		dl	2.0	1.3, 2.6	0.25
	1.5	l(-)	3.4	3.2, 3.5	0.41
	2.0	l(-)	3.3	2.2, 4.7	0.39
		dl	3.2	2.9, 3.9	0.45
Valine	0	..	-2.8	-3.1, -2.5	-0.64
	0.5	dl	-1.3	-1.6, -0.8	-0.32
		d(-)	-0.3	-0.7, +0.3	-0.05
	0.7 ¹	dl	+1.6	+1.4, 1.8	+0.16
		l(+)	3.8	2.0, 4.7	0.35
	1.0	dl	1.3	0.9, 1.8	0.18
	1.5	dl	3.4	1.9, 5.1	0.42
	2.0	dl	3.3	2.2, 4.7	0.39

¹ Results obtained from the second experiment.

Isoleucine

As the level of dl-isoleucine in the diet was increased the growth rate increased rapidly until a rate of 4% per day was obtained when 1% of isoleucine was fed, after which the rate decreased slightly when the isoleucine content was increased to 2%. In the second experiment the growth rates obtained with equal levels (0.45%) of dl-, d(—)-, and l(+)- isoleucine were compared. The data show (table 3) that d(—)- isoleucine cannot be utilized for the promotion of growth in the young chick and that the l(+) form is twice as effective as the dl form in this regard. The level chosen allowed the maximum differences in growth between the three forms. These data indicate that the requirement for l(+) isoleucine is approximately 0.5% of the diet since the maximum growth was obtained at 1% dl-isoleucine.

Leucine

In the absence of leucine, the average loss per day was 2.7%. As the leucine content of the diet was increased, growth increased until, at the 1.5% level of l(—) leucine, the growth was 3.4%. From the growth rates as shown in table 3, it is seen that the l(—)-leucine requirement is approximately 1.5% of the diet. The growth rates with 1% and 2% of dl-leucine appear to be equivalent to those obtained with equal levels of l(—)-leucine. (If only l(—)-leucine were utilizable for growth, the expected gain with 1% dl-leucine would be about —1%, while 2% dl-leucine would allow a growth rate of only 2%).

Valine

Growth at the 1.5% level of dl-valine was as good as that obtained with 2% of the dl-form. When the optical isomers were fed, an average gain of 3.8% was obtained for the 10-day period with 0.7% l(+)-valine, a loss of weight (0.3% per day) occurred with 0.7% of d(—)-valine, and an intermediate value (1.6%) was obtained with 0.7% dl-valine. From these results, the requirement is estimated to be 0.7% of the natural form.

The chick is able to utilize only the natural forms of iso-leucine and valine for growth, and in this respect is the same as the mouse (Bauer and Berg, '43) and the rat (Rose, '38). The metabolism of leucine in the chick, on the other hand, appears to be different from that in the rat and the mouse, since only the natural form is utilized for growth in the latter animals. This is the only instance which has been found thus far in which the requirements of the chick are less specific than those of other animals.

SUMMARY

Use of a diet containing mixtures of amino acids has shown the requirements of the chick for best growth to be: l(+)-iso-leucine, approximately 0.5% of the diet; l(—)-leucine, 1.5%; and l(+)-valine, 0.7%. d(—)-Isoleucine and d(—)-valine are not utilized for growth, but dl-leucine appears to be as effective as l(—)-leucine in promoting growth.

ACKNOWLEDGMENTS

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THE PANTOTHENIC ACID CONTENT OF THE BLOOD AND TISSUES OF THE CHICKEN AS INFLUENCED BY THE LEVEL IN THE DIET

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The effect of the amount of the various B vitamins in the diet on the amounts in the tissues of animals normally used for human food has been studied to only a limited extent. The thiamine content of pork tissue has been shown by several workers (Hughes, '41; Miller et al., '43; and Heinemann et al., '46) to be related to the amount ingested.

While information is available on the amounts of various B vitamins in the egg, very little is known about the influence of the amount of any of the B vitamins in the diet on the level in the tissues and blood of the chicken. Snell, Pennington and Williams ('40) fed four chicks on a diet furnishing an adequate amount of pantothenic acid and four on a diet containing an insufficient amount. They found that the pantothenic acid content of the tissues of 26-day-old chicks was influenced by the amount of pantothenic acid in the diet. Pearson, Melass and Sherwood ('45) have shown that the level of pantothenic acid in the diet determines to a large extent the concentration in the egg, but that irrespective of the amount at the beginning of incubation, the amount in the egg changes very little during embryonic development. The pantothenic acid content of the blood of various mammalia and its distribution between plasma and cells has been reported by Pearson ('41). No comparable information is available for the chicken.

This investigation was carried out to obtain information on the influence of the amount of pantothenic acid in the diet of the chicken on the levels in the tissues and the blood.

EXPERIMENTAL

Methods

The dietary treatment of the chickens was essentially the same as described in a previous publication (Pearson et al., '45). The hens used in these experiments were chiefly White Leghorns. They had been fed the experimental diets, which differed only in respect to the amounts of pantothenic acid, for a period ranging from 12 to 24 months. The two levels of pantothenate in the diets were 385 μg and 1575 μg per 100 gm of feed.

The blood for analyses was drawn directly from the heart into an oxalated tube. Pantothenic acid assays were made on the whole blood, the plasma and cells. The plasma and cells were separated by centrifuging the whole blood for 50 minutes at approximately 2500 r.p.m.

Pantothenic acid assays were made on the liver, the breast tissue and the leg muscle. Pantothenic acid was determined by the microbiological method of Neal and Strong ('43) after liberation of the vitamin by autoclaving and enzymatic treatment essentially as described for newly hatched chicks (Pearson et al., '45).

Blood studies

The whole blood of chickens on the 1575 μg level of pantothenic acid, which may be considered approximately adequate to meet the dietary requirements, contained an average of 43.6 μg of pantothenic acid per 100 ml. This is of the same order as the values for mammalia (Pearson, '41) which ranged from 19.4 μg for human blood to 71.7 μg per 100 ml of blood for rabbits. In these same studies horse blood was found to contain an average of 44.8 μg of pantothenic acid per 100 ml.

The level of pantothenic acid in the whole blood, the plasma and the cells (table 1) is definitely influenced by the amount of

pantothenic acid in the diet. The pantothenic acid levels for the hens on the 1575 μg level were, for whole blood 43.6 μg , for plasma 51.6 μg , and for cells 21.9 μg per 100 ml, respectively, as compared to 20.0 μg , 20.9 μg , and 13.0 μg per 100 ml, respectively, for the hens on the 385 μg level of pantothenic acid. The percentage difference between the values of the two groups are 118 for whole blood, 147 for plasma and 40.6 for the cells. The mean differences between the pantothenic acid values for the whole blood, plasma and cells of the hens on the two different dietary levels are each highly significant according to Fisher's *t* test.

TABLE 1

The pantothenic acid content of chicken blood as influenced by amount in the diet.

PANTOTHENIC ACID IN THE DIET	NO OF BIRDS	CELLS		BLOOD		PLASMA		CELLS	
		Mean	S.D. ¹	Mean	S.D.	Mean	S.D.	Mean	S.D.
		Vol. %		$\mu\text{g}/100\text{ ml}$		$\mu\text{g}/100\text{ ml}$		$\mu\text{g}/100\text{ ml}$	
385 $\mu\text{g}/100\text{ gm}$	17	26.8	3.7	20.0	6.9	20.9	8.5	13.0	2.6
1575 $\mu\text{g}/100\text{ gm}$	17	27.3	3.4	43.6	9.7	51.6	9.5	21.9	6.2

¹ Standard deviation.

The data on the effect of the level of dietary pantothenic acid on the level in the blood of chickens are in accord with the observation (Silber, '44) that dogs depleted of pantothenic acid have less pantothenic acid in their blood than the blood of dogs on stock diets.

There was no significant difference in the volume per cent of cells in the blood of the hens fed the high and low levels of pantothenic acid.

On the basis of the level of pantothenic acid found in the plasma and cells, the calculated amount for whole blood, as shown in table 2, agrees fairly well with the observed or determined value for whole blood (table 1). The percentage distribution of pantothenic acid between the plasma and cells based on the values per 100 ml each of cells and plasma has been calculated and is presented together with the per cent cells in table 2. The table shows that 86.2% of the pantothenic

acid of the blood of the hens fed the 1575 μg level occurs in the plasma, whereas in mammalia the per cent of pantothenic acid that occurs in the plasma ranges from 44.3 to 62.6 (Pearson, '41).

TABLE 2

Calculated amount of pantothenic acid in whole blood and its distribution between plasma and cells.

PANTOTHENIC ACID IN DIET	CALCULATED AMOUNT IN WHOLE BLOOD ¹	VARIATION FROM DETERMINED VALUE FOR WHOLE BLOOD	PER CENT OF TOTAL P.A. IN WHOLE BLOOD OCCURRING IN:	
			Plasma	Cells
	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$		
385 $\mu\text{g}/100\text{ gm}$	18.7	- 1.4	81.7	18.3
1575 $\mu\text{g}/100\text{ gm}$	43.5	- 0.1	86.2	13.8

¹ Calculated amount in whole blood — per cent cells times pantothenic acid per 100 ml of cells plus per cent plasma times pantothenic acid per 100 ml of plasma.

Tissue studies

The influence of the level of pantothenic acid in the diet on the content in tissues is shown in table 3. The liver of the hens on the low pantothenic acid intake contained an average of 40.7 μg of pantothenic acid per gram of fresh tissue as compared with 44.9 μg for the livers of the hens on the high intake.

TABLE 3

The effect of the amount of pantothenic acid in the diet of the chicken on the amount in the tissues.

PANTOTHENIC ACID IN DIET	NO. OF BIRDS	LIVER		LEG MUSCLE		BREAST TISSUE	
		Mean	S.D. ¹	Mean	S.D.	Mean	S.D.
		$\mu\text{g}/\text{gm}$		$\mu\text{g}/\text{gm}$		$\mu\text{g}/\text{gm}$	
385 $\mu\text{g}/100\text{ gm}$	15	40.7	7.8	8.4	1.9	6.5	1.2
1575 $\mu\text{g}/100\text{ gm}$	15	44.9	6.9	17.2	2.8	11.3	3.2

¹ Standard deviation.

According to Fisher's *t* value there is no significant difference in these two values. The similar pantothenic acid values for the livers of chickens on different dietary levels is in contrast to the findings of Silber ('44) with dogs. This investigator

reported that the livers from dogs depleted of pantothenic acid contained significantly less pantothenic acid than the livers of dogs fed a stock diet. On the other hand, Heinemann et al. ('46), working with pigs, fed different levels of riboflavin without influencing the riboflavin content of the liver. Furthermore, the subsequent feeding of a diet containing approximately 3950 μg of pantothenic acid per 100 gm did not further increase the amount in the liver of chickens. The data suggest a species difference between the dog and the chicken with respect to the influence of the amount of pantothenic acid in the diet on the concentration in the liver.

The pantothenic acid was determined on livers from eight chickens 9 weeks of age that had been fed a stock diet from the time of hatching. The average pantothenic acid value for the livers from the chickens 9 weeks of age was 43.1 μg per gm of fresh tissue, which is not significantly different from the values reported here for older chickens. From this it appears that age is not a factor in the amount of pantothenic acid in the liver of chickens.

The level of pantothenic acid in the leg muscle is higher than in the breast tissue. The difference between the level of pantothenic acid in the leg muscle and the breast tissue is highly significant for both the high and low level of pantothenic acid intake.

The leg muscle of the chickens on the high pantothenic acid intake contained an average of 17.2 μg per gm of fresh tissue as compared with an average of 8.4 μg for the group on the low intake. The breast tissue of the chickens on the high pantothenic acid level contained an average of 11.3 μg per gm as compared with 6.5 μg of pantothenic acid for the chickens on the low level. The differences between the values for the two levels of pantothenic acid are highly significant for both the leg muscle and breast tissue. Similarly the amount of pantothenic acid in the diet of dogs was found by Silver ('44) to influence the amount of pantothenic acid in the leg muscle.

The rate at which depleted tissues replenish their store of pantothenic acid was studied by changing hens from the 385

μg level of pantothenic acid to the 1575 μg level. One group of four chickens was killed at the end of 14 days and a second group after 28 days, and the tissues assayed for pantothenic acid. At the end of the 14-day period the average pantothenic acid content of the leg muscle was 17.9 μg per gm, which is essentially the same as for the chickens that had continuously been on the high intake. However, the average value for the breast tissue at the end of 14 days was only 8.8 μg as compared with 11.3 μg of pantothenic acid per gm. These data indicate that the rate of storage of pantothenic acid is more rapid in the leg muscle than in the breast tissue of the chicken. After 28 days on the high intake the pantothenic acid content of the breast tissue had increased to the same order as for birds that had continuously been on the high intake and there was no significant change from the 14-day-period in the concentration of pantothenic acid in the leg muscle.

In order to determine if the pantothenic acid content of breast tissue and leg muscle could be increased above the level for the 1575 μg level of intake, the amount of pantothenic acid in the diet of some of the chickens from this group was increased to approximately 3950 μg per 100 gm of feed. Four weeks later the levels of pantothenic acid in the tissues of the chickens were not significantly different from the average for the birds kept continuously on the 1575 μg level.

SUMMARY

Data are presented on the effect of the amount of pantothenic acid in the diet of chickens on the level in the blood and tissues.

On an adequate intake of pantothenic acid (1575 μg per 100 gm of feed) the average level in whole chicken blood was 43.6 μg per 100 ml and for plasma 51.6 μg . These values are 118 and 147% higher than the respective values for the blood and plasma of chickens on an intake of about 385 μg of pantothenic acid per 100 gm of feed. In whole chicken blood an average of 86.2% of the pantothenic acid occurs in the plasma, which is

somewhat higher than the corresponding figure for the blood of mammalia.

The amount of pantothenic acid ingested had no significant effect on the amount of pantothenic acid in the liver. On a diet containing approximately 1575 μg of pantothenic acid per 100 gm of feed the average pantothenic acid value for liver was 44.9 μg , for leg muscle 17.2 μg and for breast tissue 11.3 μg per gm of fresh tissue. The level of pantothenic acid in the leg muscle and breast tissue was significantly influenced by the amount ingested. The addition of pantothenic acid to the diet of chickens that had been on a low intake resulted in a relatively rapid deposition of pantothenic acid in the leg muscle and breast tissue so that the amount in these tissues reach the normal level for the amount ingested within a period of 2 to 4 weeks.

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source. Stefaussen ('44) believes that as much as 80% of the calories may come from fat.

In view of these observations which have extended over many years and many cultures, it is extraordinary to read of the results of recent experiments upon diets consisting exclusively of protein and fat (Kark, Johnson and Lewis, '45) in which it was clearly shown that the subjects upon these diets were not only incapable of hard work but even appeared to be no better off than if they had gone without food altogether. This sharp discrepancy between well established facts and recent experiments has puzzled us greatly. The most obvious difference between the two sets of observations is that the diets in the earlier work consisted of meat and fat eaten either raw or after ordinary cooking, while in the work of Kark, Johnson and Lewis ('45) pemmican was used. The pemmican was prepared by dehydrating cooked lean meat, grinding it up and then mixing it with melted fat. There is nothing in this process which would be expected to change the nutritional properties of the food (except the flavor) and yet there appeared to be a very great change in these properties. Could it be the dehydration? This seemed unlikely as there was no limitation of the water intake and no reason to suppose the protein was not rehydrated in the digestive tract. The subjects in Kark's experiments were only on the diet for 3 days. Was this too short a time to accustom them to the diet? To answer these and other questions we carried out a short preliminary study with twelve men on a field trip.

METHODS

A field trial was held for 3 weeks on a small island, Pasque, near Woods Hole, Buzzards Bay, Mass. The temperature ranged from 48°F. to 70°F., the weather being rainy and misty most of the time. The subjects, including three civilians and nine enlisted men, were laboratory workers, performing the qualitative and quantitative biochemical analyses essential to the study. The group was divided so that eight men acted as subjects and four men as controls. The controls ate a good

packaged ration supplemented with bread, butter, jam, coffee, tea, and extra evaporated milk and sugar, and their daily intake of nutrients was, in all respects, at least as high as the standard recommended by the National Research Council. Their average protein intake was 105 gm per day, and fat accounted for 30% of their caloric intake. The experimental subjects ate pemmican, made by adding 50% of fat to 50% of ground dried lean beef, with 1% salt added. On such a diet, 71% of the calories are derived from fat, 27% of the calories from protein, and 2% from carbohydrate. They had the choice of either tea or coffee without sugar or milk, and as much water as they wanted to drink. There was an initial period of 5 days on a normal diet, followed by 9 days' experimental period on pemmican and then a return to a normal diet for 4 days. Biochemical tests were made during all three periods.

On the first day of the diet, the subjects were each given a can of pemmican that weighed approximately 460 gm, containing 7 cal. per gm. They were cautioned to eat only 100 to 200 gm the first day or so and then only when really hungry. The reason for this warning was previous experience in which many subjects on changing from a normal diet to a high fat diet became nauseated and vomited during the first 2 days. A careful check was kept on the daily food consumption of each subject.

Biochemical and physiological measurements were made periodically. Body weight was measured daily. Fluid balance was calculated daily from body weight, fluid intake and fluid excretion. Physical fitness was measured by the "pack test" (Darling et al., '44; Johnson, Brouha and Darling, '42). Basal metabolic rate was measured by a Douglas bag method. Biochemical measurements and methods included: serum protein, Phillips et al. ('43); blood glucose, Folin and Malmros ('29); serum and urine chloride, Keys ('37); serum non-protein nitrogen, Daly ('33); serum cholesterol, Bloor ('26); serum and urinary ascorbic acid, Farmer and Abt ('36); urinary nitrogen, Ma and Zuazaga ('42); urinary riboflavin, thiamine and N¹-methylnicotinamide, Johnson, Sargent, Robinson and

Consolazio ('45); urinary ketone bodies, qualitative, Rothera (see Hawk and Bergeim, '37); bromsulfalein test of liver function, Rosenthal and White ('25); phenolsulfonphthalein test of kidney function; oral glucose tolerance test, Exton and Rose ('34); insulin tolerance test. Laboratory procedures were conducted by means of our portable field laboratory, Johnson ('45).

RESULTS

For the first day or so the subjects ate the pemmican warmed slightly, but this method was given up as the dehydrated meat had to be chewed for quite a long time, which made one nauseated. On the third day, one of the subjects found that after addition of water and boiling the mixture for $\frac{1}{2}$ to 1 hour, it

TABLE 1
Daily intake of pemmican in grams.

SUBJECT	DAY OF DIET									TOTAL
	1	2	3	4	5	6	7	8	9	
M.C.	139	135	191	172	55	89	126	53	138	1093
F.C.	190	183	398	238	230	231	90	143	67	1770
W.F.	140	208	181	195	90	66	162	220	73	1335
P.K.	113	160	148	191	100	50	31	4	0	787
A.R.	81	61	49	39	0	0	0	0	0	230
G.S.	121	374	245	670	635	467	662	510	408	4093
J.S.	138	184	177	321	241	190	130	49	64	1494
R.W.	162	211	155	128	120	10	0	0	0	786
Ave.	136	190	193	244	184	138	150	122	94	1449

made a fair stew. This seemed to be the best way to eat the pemmican, for the amount consumed kept increasing daily up to the fourth day, to 244 gm per person (table 1). After the fourth day the amount decreased gradually, so that on the ninth and last day the average consumption was 94 gm (660 cal.). One subject, A. R., became nauseated on the first day and as a result ate only 230 gm during the whole experimental period. He felt that he would rather starve the last 5 days than run the risk of vomiting. Another subject, G.S., was quite the opposite. He ate about 200 gm per day for the first

3 days, and then ate from 400 to 674 gm daily (2800 to 4720 cal.) the remaining 6 days. For the last 3 days he ate from 50 to 150 gm more than the rest of the group put together. At the end, no subject had begun to enjoy the taste of the food nor to feel that he could live on it.

During the first few days morale was fair but deteriorated noticeably until the subjects became listless, disheartened and difficult to rouse to do any physical work. They preferred to avoid strenuous exercise, particularly if it was prolonged, but did quite well on the physical fitness test (lasting only 5 minutes), which everyone was compelled to do on every third day.

Physical fitness in the subjects, as measured by the "pack test," showed an increase in score from 65 to 74, during the first 2 days and then leveled off until the end of the diet. After 3 days on a normal diet, the score increased from 74 to 83. The controls, on the other hand, increased gradually from a score of 46 before the diet to 73, 3 days after the diet. The improvement during the first few days of the experiment is explained largely by the fact that most of the men were not in training at the beginning of the experiment. The constancy of the scores during the dietary period is misleading because the men were 5.9 kg lighter at the end of the period and no extra weights were added to the packs to compensate for their weight loss. Such a decrease in work done should be accompanied by a definite increase in score, whereas no increase in score was found. The controls improved continuously during the dietary period, which suggested that the control diet was definitely better.

Body weight showed striking changes, the experimental subjects averaging 5.9 kg loss in weight as compared with the 1.0 kg lost by the controls (table 2). In 3 days of normal diet the experimental subjects regained 3.6 kg. Part of these changes were on a caloric basis because of the average daily caloric deficit of about 1500. Part was also due to change in water balance. In the subjects during the first 3 days on the diet more fluid was put out in the urine than was taken in by mouth; in the controls the intake always exceeded the urinary output

TABLE 2

Changes in body weight during and after pemmican.

SUBJECTS RECEIVING PEMMICAN	BEFORE		DAYS DURING					DAYS AFTER	
	1	2	4	5	7	9	1	3	
M.C.	74.8	72.1	71.6	70.9	70.1	69.1	70.5	72.9	
F.C.	95.0	92.1	90.6	90.5	89.5	87.7	90.3	90.8	
W.F.	73.4	70.0	69.1	68.2	67.5	66.4	68.0	68.7	
P.K.	75.0	72.7	72.2	71.3	70.0	69.0	71.9	72.9	
A.R.	89.8	87.2	85.5	85.0	83.8	82.5	84.4	85.6	
G.S.	65.9	62.8	64.2	63.1	63.7	62.9	64.5	66.3	
J.S.	74.0	70.7	69.9	69.6	68.7	68.0	71.1	72.9	
R.W.	66.7	65.4	64.2	64.2	61.9	60.4	62.5	65.0	
Ave.	76.7	74.1	73.4	73.0	71.9	70.8	72.9	74.4	

Controls

J.E.	68.2	66.9		66.5		66.2	67.5	67.8
F.M.	79.7	79.6		79.7		79.2	79.4	79.2
J.P.	69.0	68.2		68.7		68.3	68.5	68.6
S.T.	64.3	65.0		64.6		64.1	64.2	64.8
Ave.	70.4	69.9		69.6		69.4	69.8	70.1

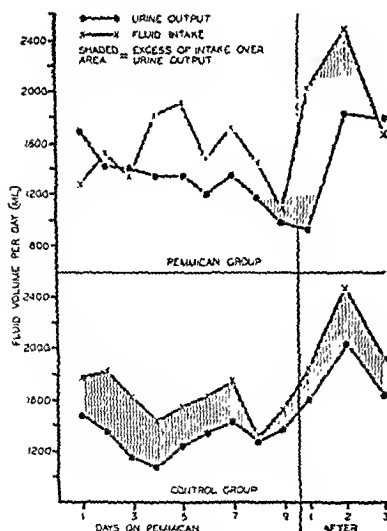


Figure 1

Fig. 1 Daily fluid intake and urine output.

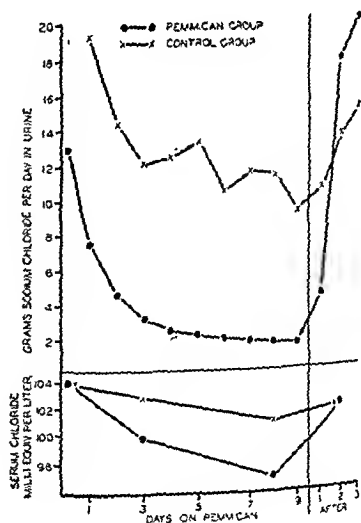


Figure 2

Fig. 2 Serum and urine chlorides.

(fig. 1). At the same time the urinary excretion of ehloride (fig. 2) and its serum level were dropping steadily in the subjects but not in the controls. For 2 days after resumption of normal diet, there was a large retention of water by the subjects with increasing serum ehloride and urinary excretion of ehloride.

TABLE 3

Measurements showing no significant difference between pemmicon group and controls.

MEASUREMENTS AND UNITS	SUBJECTS	PERIOD OF TRIAL			
		Before 1 day	Experimental ration 4 days	9 days	After 3 days
Serum N.P.N. mg/100 ml	Pemmican	35	40	32	28
	Controls	35	28	30	27
Serum cholesterol mg/100 ml	Pemmican	190		205	175
	Controls	165		210	195
Serum ascorbic acid mg/100 ml	Pemmican	0.6	0.4	0.5	0.4
	Controls	0.3	0.2	0.4	0.4
Serum protein gm/100 ml	Pemmican	6.7	6.8	6.7	6.3
	Controls	6.5	6.6	6.2	6.5
Urine ascorbic acid mg/hour	Pemmican	0.8	0.6	0.2	0.5
	Controls	0.5	0.5	0.1	0.3
Urine thiamine μg/hour	Pemmican	11	7	7	6
	Controls	8	6	6	6
Urine riboflavin μg/hour	Pemmican	76	41	32	53
	Controls	63	46	31	42
Fasting blood glucose mg/100 ml	Pemmican	101	112	103	107
	Controls	100		107	112
Basal metabolic rate % of normal	Pemmican	+ 6		+ 9	0
	Controls	+ 5		+ 6	0
P.S.P. kidney function % ex- creted in 2 hrs.	Pemmican	64		69	61
	Controls	68		66	73

Of the many other measurements made, some, discussed later, showed differences between the experimental subjects and the controls, and the rest did not. The latter are listed in table 3 and included: serum NPN, serum cholesterol, serum

ascorbic acid, serum protein, fasting blood sugar, BMR, kidney function tests, urinary ascorbic acid, thiamine and riboflavin. Significant differences were found in urinary ketone bodies, nitrogen, N¹-methylnicotinamide and the tests of glucose tolerance, insulin tolerance and liver function. These will be discussed below.

Beginning on the first day of the diet, the subjects had a ketosis, a two-plus positive nitroprusside reaction in the urine which increased to a four-plus from the fifth to the ninth days, and it was not until the third day after the diet that they showed a negative nitroprusside reaction again. This ketosis was shown equally by the subjects who ate the most and the least (G.S. and A.R.).

The excretion of N¹-methylnicotinamide increased in the experimental subjects from 0.44 mg per hour to 0.96 mg per hour but the controls stayed practically constant at 0.32 before to 0.42 mg per hour during the experimental period. It was not an effect of starvation as shown by subject G.S. who kept in caloric balance, but was probably due to the large intake of protein.

The urinary excretion of nitrogen averaged 173 gm (1080 gm protein) per person for the 9-day period, the highest daily output being 24.4 gm and the lowest 14.2 gm, which was still high. On the other hand the average nitrogen intake was 103 gm per person (644 gm protein) which was a deficit of 70 gm. nitrogen during the whole experimental period. Thus even though the subjects took in reasonable amounts of protein (average 50 gm/day omitting subject G.S. or 69 gm/day including G.S.), all except G.S. still burned their body protein at the average rate of 49 gm per day to help supply their caloric deficiency. The only subject (G.S.) who did not have a caloric deficiency ate 265 gm of protein per day and did not use any of his body nitrogen; in fact he had a positive nitrogen balance.

There were significant changes in both the insulin and glucose tolerance tests (tables 4 and 5). During the period of the diet the blood glucose of the pemmican group did not fall to abnormally low levels either in the basal state or after 2.5

units of crystalline zinc insulin were injected intravenously, but the depression after insulin was definitely prolonged. The most interesting part of the insulin tolerance tests was the reactions that the subjects showed during the dietary period. Whereas no one had any marked symptoms before or after the diet, five of the eight men who had insulin tolerance tests

TABLE 4
Insulin tolerance tests.

GROUP	BLOOD GLUCOSE (MG/100 ML)						
	Fasting	20 min.	25 min.	30 min	35 min.	45 min	60 min.
<i>Pemmican</i>							
Before diet	111	83	80	83	93	106	105
Test period	107	77	74	70	80	85	85
After diet	119	83	79	85	87	106	107
<i>Control</i>							
Before diet	100	76	80	88	92	93	96
Test period	108	73	75	83	90	102	100
After diet	112	66	72	86	96	105	106

TABLE 5
Glucose tolerance tests. (All subjects received 75 gm of glucose).

TIME OF TEST	BLOOD GLUCOSE (MG/100 ML)					BLOOD GLUCOSE (MG/100 ML)				
	Fast-ing	30 min	60 min	120 min.	180 min	Fast-ing	30 min.	60 min	120 min	180 min.
<i>Pemmican</i>						<i>Control</i>				
Before diet	99	168	168	117	102	100	159	145	136	100
Test period	108	173	186	128	104	110	146	135	117	100
After diet	106	159	135	110	113	121	180	148	124	103

during the diet complained of faintness, light headedness, dizziness, hunger pains and nausea. The subjects became pale, sweated considerably and were noticeably glassy-eyed. Each subject received 2.5 units of crystalline zinc insulin intravenously, except W.F. and P.K. who both received 1.6 units. Even with such small doses W.F. had quite a bad reaction, which included nausea, faintness and hunger pains.

The results of the glucose tolerance tests were a surprise to all, for we had expected to see the subjects, on being given the glucose solution during the period on this diet, perk up and become happy and active. Instead, no subject detected any beneficial effects. The blood sugar curves during the glucose tolerance tests were significantly higher in the pemmican group.

TABLE 6
Liver tolerance test.¹

TIME OF TEST	DYE RETAINED (%)			DYE RETAINED (%)		
	15 min.	30 min.	45 min.	15 min.	30 min.	45 min.
	<i>Pemmican</i>			<i>Control</i>		
Before diet	29	12	5	21	9	7
Test period	50	20	12	30	10	5
After diet	29	9	6	24	6	5

¹ 5 mg Bromsulfalein intravenously per kg of body weight.

The ketonuria, which did not disappear until 3 days after the diet was caused not only by starvation but also by the high fat and very low carbohydrate diet. This was shown very clearly during a glucose tolerance test in two different subjects. The nitroprusside reaction was a four-plus before drinking 75 gm of glucose in solution but in 1 hour in one case and 2 hours in another the reaction was negative. We will discuss the ketonuria again later in the paper.

The liver function test during the test period showed a significant increase in the retention of the dye at 15, 30 and 45 minutes (table 6).

DISCUSSION

For practical purposes, the present work supports the contention that pemmican is unsuitable for a field ration if used as the sole component.

At the beginning of the experiment, all the subjects seemed sure that they would have no trouble eating the pemmican, for at first it did not taste too badly when eaten slightly warmed and in small amounts, but this method was soon given up be-

cause it began to be very nauseating. The idea of eating it in the form of a stew also seemed to be good, but here again the novelty wore off within 2 days because the taste of the meat became more and more repulsive as days went by and as a result the amount consumed per person decreased gradually so that on the last day the average consumption was 94 gm (660 cal.).

Kark, Johnson and Lewis ('45) made a thorough search of the literature of explorers in cold climates and reported that there was no clear evidence that men had ever lived successfully on pemmican alone. It was always supplemented with biseuits and cereals and whatever other foods were available. The pemmican was commonly saved until the end, either because it was not liked or possibly because it was the most concentrated and expensive food. Stefanssen ('44), on the other hand, states that a fair test of pemmican should take at least 2 weeks, and in that time a person will grow to like it, but we found that the pemmican was edible in only very small amounts, not sufficient to keep a man in good condition for a long time. We had hoped to acquire a taste for it as days went by but instead it became more and more difficult to eat as time went on. The one subject who ate the most had to force himself through the last half of the experimental period. However, Peary ('17) and Priestley ('15) were enthusiastic about pemmican as the major component of a ration.

Each night the subjects in response to questions concerning subjective feelings and cravings agreed that they would much rather have almost anything to eat in place of pemmican. Many mentioned carbohydrate food but meats were also mentioned occasionally and there was no clear craving for carbohydrate. All had had visions of eating enormous quantities of food on the first meal after the diet, but when this hoped for meal at last came, almost everyone complained that he was filled and satisfied too soon. The average caloric consumption of the subjects at this meal was 1380 cal. and one of the most striking features was that all men ate large amounts of bacon and butter although carbohydrate food was equally available.

This suggests that people in general will eat fat that is naturally in food, but will not eat it in the form of pemmican.

The subjects, after having lost so much weight during the diet, retained an average of 1300 ml of fluids on the first day on a normal diet. We feel that the high fat had a tendency to slightly dehydrate the men, as shown in the case of G.S., who ate large quantities of the pemmican. However, our data are not adequate to differentiate between the effects of high fat and the effects of low chloride upon the water balance. G.S. still lost 3.0 kg in the 9-day period, but all of it was water because the calculated weight change from daily caloric intake and daily expenditure was plus 0.2 kg. This would make his water loss 3.2 kg. G.S. in contrast to the other subjects had slightly more than regained his normal weight by the third day on a normal diet. The weight loss of the whole group, calculated from their estimated caloric deficiency, was 1.7 kg which subtracted from the observed loss of 5.9 kg leaves 4.2 kg due to loss of fluid. The weight regained by the whole group by the third day on a normal diet was only 3.6 kg.

The general physiological findings of the present study confirm and extend the conclusions of Kark, Johnson and Lewis ('45). The biochemical abnormalities produced in our subjects by the high fat diet even in the one who ate the most, included changes in water and salt balance, liver function and glucose and insulin tolerance tests. While the subjects showed a marked ketosis, as might well be expected on this type of diet, it is not clear that this ketosis was detrimental. Heinbecker ('28) has shown that Eskimos on a meat diet do not show ketosis. Our subjects showed no change in scores in the physical fitness tests except for the initial 2 days but their daily work output was small and caloric deficits affect physical fitness most strikingly in men who are working hard.

There was unusually high sensitivity to insulin in five of the eight subjects on pemmican. In the literature very little is said about sensitivity to insulin on a high fat diet, but Himsworth ('34a) states that on a high fat diet the sensitivity to insulin is low and that subsistence on such a diet retards

and diminishes the action of insulin upon the blood sugar. Wishnofsky, Kane and Spitz ('37) conclude that fat does not require insulin for its metabolism for it does not inhibit or retard the action of insulin. Riesser ('42) also states that a protein and fat diet produces a greater decrease in sensitivity to insulin shock than a diet consisting partly or largely of carbohydrate. Some work has been done on the sensitivity to insulin in starvation. Selye ('40) in experiments with rats, states that although fasting progressively increases the insulin sensitivity, there is a transitory period after about 2 days of starvation during which the insulin sensitivity is very low. Gigante ('35) in experiments with starving pigeons, states that the insulin resistance shows a sudden decrease at the point of hunger crisis, usually ending in convulsions. We cannot agree with Himsworth, Wishnofsky, and Riesser, for one of our subjects, G.S., who ate large quantities of the high fat diet, had a bad reaction during the insulin tolerance tests. Another subject, R.W., who practically starved the last 4 days, had reactions that were so severe that glucose had to be administered. To conclude, a high fat diet as well as a semi-starved condition, increased sensitivity to insulin as judged by the reactions other than the fall in blood sugar which fall was not increased though it was prolonged.

The glucose tolerance curves during the dietary regime which were significantly higher are in agreement with those of Himsworth ('34b), Threadwell, King, Babb and Tidwell ('42), and Greene and Swanson ('40), who all state that a high fat diet produces a decrease in sugar tolerance. Wilson ('39) states that the glucose tolerance curve has been employed successfully to detect early deficiencies in liver function. The curve in such cases is high and its fall is delayed and liver extract lowers it towards the normal.

There was increased retention of bromsulfalein dye during the experimental diet. Abnormal retention of the dye has been interpreted as evidence of liver dysfunction (Rosenthal and White, '25; Bulmer, '28; Foley, '30; Macdonald, '38; Helm and Machella, '42). There is general agreement that a

retention of over 5% at 30 minutes is abnormal. One case of Robertson, Swalm and Konzelmann ('32) showed 100% retention and the interpretation was made that an important factor may have been dehydration and decrease in blood volume. In the present experiments there is evidence of changed liver function, not necessarily dysfunction. A hypothesis to explain the findings is that on a high fat diet, the mechanisms for regulating glycogen in the liver are altered in such a way that a given dose of insulin keeps the blood sugar at a low level for an abnormally long time. The bromsulfalein test confirms the alteration of liver function.

The urinary and serum chlorides, which were significantly low at the end of the diet, showed that even though a man ate more than his share of the pemmican daily, as in the case of G.S. who ate from 2800 to 4720 cal., the salt content was insufficient to keep the average man in salt balance with a satisfactory margin of safety over a long period of time. At the end of the diet G.S. was excreting only 5.7 gm of salt per day and his serum chloride had dropped from 106 to 100 milliequivalents per liter.

SUMMARY

A group of eight men living in a cool environment and doing work consisting mostly of laboratory procedures subsisted for 9 days on a high fat diet (pemmican) providing 71% of the calories from beef fat and 2% from carbohydrate. Four controls subsisted on a diet adequate in all respects and providing 30% of the calories from fat.

The utility of pemmican alone as a field ration for ordinary men was very poor because of the inability of all but one subject to eat enough of it. Morale deteriorated on the diet and most of the men resigned themselves to semi-starvation for the duration of the diet, mainly because of the nauseating taste. Nevertheless, scores in a physical fitness test remained practically constant.

Significant biochemical and physiological changes occurred, even in the one man who ate adequate amounts of the pemmican. These included: (a) an average weight loss of 5.9 kg,

much of it water; (b) change in water balance, with loss of body water; (c) salt depletion as measured by serum and urinary chlorides; (d) marked ketonuria; (e) alteration in the glucose tolerance curve, with prolongation of the rise but without alteration of the maximum; (f) alteration in tolerance to a given dose of insulin with much increased physiological reaction and prolongation of the decrease without alteration in the minimum; (g) increased retention of bromsulfalein. All of the above abnormalities were repaired in 3 days of normal diet.

Measurements showing no significant changes include: (a) serum protein; (b) serum non-protein nitrogen; (c) serum ascorbic acid, (d) serum cholesterol; (e) fasting blood glucose; (f) urinary excretion of thiamine, riboflavin and ascorbic acid; (g) basal metabolic rate; (h) phenolsulfonphthalein test of kidney function.

This work should not be taken to apply to all high fat diets, but at this point we do not know why there appears to be a difference between a diet of pemmican and a diet of fresh meat and fat.

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THE EFFECT OF EXCESS DIETARY CALCIUM ON LONGEVITY AND TISSUE CALCIUM IN THE ALBINO RAT¹

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In a series of articles, it has been shown by Sherman and associates that the calcium content of the body of the growing rat is dependent upon the calcium content of the food supply, at least up to a content of 0.64% and possibly somewhat higher (Toepfer and Sherman, '36; Lanford, Campbell and Sherman, '41). In a later article (Briwa and Sherman, '41), it is concluded from analyses of well over 400 experimental animals sacrificed at 28 days of age "that among normal growing individuals of a given sex, having the same hereditary and nutritional background, age is the predominant determining factor in the increasing percentage of calcium in the body."

In another phase of the same series of studies, Campbell, Pearson and Sherman ('43) demonstrated an improvement in the longevity of rats receiving a diet containing 0.34% of calcium as compared with one containing only 0.20%, in both males and unmated females. Van Duyne and others ('41) claim to have shown that further increases in the concentration of dietary calcium up to 0.64, or possibly 0.80%, "gave best results in permanent nutritional well-being, as shown by life-time experiments in three generations of rats main-

¹ The investigation reported in this paper was aided by funds contributed by the American Dry Milk Institute, Inc., of Chicago, Illinois.

² Now with the Golden State Co., Limited, of San Francisco, California.

tained under laboratory conditions." The basal diet in both of these investigations was demonstrably inadequate in riboflavin and vitamin A. On a more favorable basal diet, calcium levels of 0.34, 0.48 and 0.64% induced equally good records throughout the life histories of the experimental animals (Campbell and Sherman, '45).

The experiments thus briefly reviewed are of fundamental importance in nutrition. If they may be criticized at all, it may be on the score that the feeding of a constant diet throughout the life of an animal is not a sufficiently realistic nutritional endeavor if the results obtained are to be applied to human nutrition. Infants, children and adults are not nourished in this manner, nor is such a system one that can be recommended because of the changing nutritive requirements with age and with such functions as growth, reproduction and lactation. Specifically, these experiments furnish no information on the very practical question whether in adult life, and regardless of the special nutritive requirements of gestation and lactation, any advantage, or any disadvantage, accrues from an intake of calcium obviously in great excess of current requirements. Thus, an adult rat may be brought into calcium equilibrium on a diet containing only 0.03% of calcium³ so that the requirement for continued adult maintenance can hardly be much more than this.

The tissue changes accompanying senescence in man seem to involve rather generally an increase in calcium content (Simms and Stolman, '37) particularly in the hyaline cartilage (Falconer, '38). Sclerosis of the large arteries involves deposition of calcium salts similar in composition to the salts of bone (Meeker and Kesten, '36), while deposition of calcium phosphates in the soft tissues accompanies many types of senile degeneration and disease (Fronzel and Prien, '46). Such age changes have been noted in the tissues of rats also by Barnes ('42). Their occurrence is accentuated by retardation of growth (Hummel and Barnes, '38), by the administration of massive doses of irradiated ergosterol (Ham and Lewis, '34).

³ According to data secured in this laboratory by Dr. John B. Longwell.

and by an excess of calcium and phosphorus in acid-producing diets (Stephens and Barr, '33).

It seemed important to study further the effect of high-calcium diets, especially in adult life, on the occurrence of soft-tissue calcification, and particularly on longevity in the albino rat. If senile degeneration with its concomitant calcification of the soft tissues is dependent solely upon the local initiation of metabolic changes, then an intake of dietary calcium in great excess of current needs will create no hazard to health and will not shorten life. But if these degenerative changes are accelerated by an excessive intake of calcium, then the calcium intake should be lowered in old age and such high-calcium foods as milk and dairy products should be consumed in moderation. It was the purpose of the investigation to be described in this report to throw further light upon this highly practical problem in adult nutrition.

PLAN OF THE EXPERIMENT

Twenty-four trios of rats, twelve trios of male rats and twelve trios of female rats, were fed from shortly after weaning (initial weights of 33 to 64 gm, averaging 45 gm) to natural death on three experimental diets designed to contain liberal amounts of all essential nutrients and to differ only in their contents of calcium and of phosphorus. These differing contents of the diets in the two mineral elements were secured by adding appropriate amounts of calcium carbonate or of dicalcium phosphate to a basal diet consisting of casein 17, dried liver 5, dried yeast 10, salts⁴ 2, corn oil 10, sucrose 10, and whole wheat 46. After 43 weeks, the casein in this basal diet was reduced from 17 to 5% and the whole wheat raised from 46 to 58%.

During the first 43 weeks of feeding, Diet A consisted of the basal ration supplemented with calcium carbonate to raise the calcium content to approximately 0.6%. Diets B and C included supplements of dicalcium phosphate to raise the

⁴ A Ca- and P-free mixture containing all of the other mineral elements known to be essential in animal nutrition.

calcium content to approximately 1.0%. At the end of 43 weeks, the supplement of calcium carbonate was eliminated from Diet A. The calcium content of Diet B was reduced to approximately 0.7%, using a calcium carbonate rather than a calcium phosphate supplement. Diet C was unchanged. Throughout the experiment each rat received 3 drops of cod liver oil daily and 3 drops of wheat germ oil weekly.

The contents of calcium, phosphorus and ash in the three diets and in the two divisions of the experiment, by actual analysis, are summarized in table 1. It is evident that the rats on Diet A were raised on an adequate but not excessive

TABLE 1
Chemical composition of the experimental diets.

PERIOD COVERED	DIET A			DIET B			DIET C		
	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash
	%	%	%	%	%	%	%	%	%
First 43 weeks	0.59	0.56	4.15	0.98	0.86	5.45	0.98	0.86	5.45
Remainder of experiment	0.22	0.48	3.52	0.69	0.51	4.58	1.06	0.85	5.26

plane of calcium nutrition, and then when growth was completed, or shortly thereafter, the plane of calcium nutrition was lowered in conformance with lowered requirements. The rats on Diets B and C were raised on an excessive plane of calcium nutrition (about 1% in the diet). In early adult life, the calcium level of the rats on Diet B was lowered to about 0.7%, while that of the rats on Diet C was maintained at the 1.0% level throughout life. The latter rats were, thus, fed excessive amounts of calcium throughout life, especially during adulthood.

The rats were litter mates in 18 of the 24 trios, and in all trios were selected for practical equality in initial body weight. Within each trio, one rat received Diet A, one Diet B and one Diet C. Trio mates were fed equal amounts of organic matter in their respective diets, due account being taken of differences between diets in content of ash, until the death of one of the trio mates. The remaining two rats were then continued

as a pair with equal intakes of organic matter until one died, when the remaining rat was continued on an amount of food chosen voluntarily. This plan of feeding was adopted in an attempt to secure a more exact comparison of the three calcium regimes, uncomplicated in so far as possible by differences in the consumption of calories, protein, etc. If these differences had been allowed to develop, whether as subsidiary effects of the experimental diets or merely as expressions of individuality among the members of the same diet groups, the results secured could not have been so surely attributed to the imposed differences in calcium nutrition and the experimental error in average data, originating in the uncontrolled factors of the investigation, could not have been kept at a minimum, or so it seemed.

Throughout the experiment, the rats were kept in an air-conditioned room, maintained at a temperature of $80 \pm 2^{\circ}\text{F.}$, and a relative humidity of $50 \pm 5\%$.

It is realized that the chemical composition of the bodies of animals allowed to live until natural death may be determined not only by the dietary regime upon which they have subsisted, but also upon the nature and the duration of the terminal illness. For this reason, a study of the effect of diet upon the life span and a study of the effect of diet upon tissue composition are to a degree mutually incompatible. However, it was considered worth while to determine the calcium content of certain selected tissues of some of the experimental rats after natural death, as well as the ash content of selected bones. The effect of morbidity on these results will presumably be minimized if they are expressed on the moisture-free basis, or, in the case of the bones, on the moisture- and fat-free basis.

EXPERIMENTAL RESULTS

Rate of growth

The rats in all diet groups increased in body size at a satisfactory rate considering the type of dietary control imposed in order to permit the most significant comparison of the

three dietary regimes. The average data summarized in table 2 show that mature weights were attained in from 40 to 60 weeks. They also reveal no significant differences in rate of growth among the three diet groups within the two sexes. The males continued to increase slowly in body weight up to the one hundredth week to an average weight of 330 to 340 gm, presumably due to fat deposition, and then, as a group, decreased rather precipitously until death. The females maintained an average body weight of 200 to 240 gm from the

TABLE 2

Average body weights in grams at different periods in the experiment.

TIME INTERVAL AFTER:	DIET A	DIET B	DIET C	TIME INTERVAL AFTER:	DIET A	DIET B	DIET C
<i>weeks</i>	MALES			<i>weeks</i>	FEMALES		
10	197	202	199	10	158	163	160
20	246	254	249	20	188	188	186
30	282	288	279	30	199	201	193
40	278	293	280	40	205	207(11) ¹	203
50	287(11) ¹	296	290(10) ¹	50	195	204(11) ¹	194
60	311(9) ¹	315(11) ¹	287(10) ¹	60	208(11) ¹	215(7) ¹	203(10) ¹

¹ The numbers in parentheses denote the number of rats surviving at the indicated time when the original group size of 12 has been reduced by mortality.

thirtieth to the ninetieth week of the experiment, before the pre-mortal decrease in average weight supervened. The females on Diets B and C exhibited a greater average tendency to fatten in adult life than the females on Diet A, but the significance of these changes in group average weights in later life is obscured by the decreasing size of groups due to increasing mortality.

In table 3 are given the average body weights of the two surviving members of the trios in both sexes at the time of death of the first member of the trio. In ten of the twenty-four trios, the first death occurred in the rat subsisting on Diet C; in eight trios, the first death occurred in the rat on Diet B; and in six it occurred in the rat on Diet A. The average body

weights of the remaining two rats in each trio at the time of death of the first trio mate are not greatly different between the diet groups involved, although the weight advantage on the average favored in each case the rat on the more liberal calcium regime. Only one of these diet differences was sufficiently consistent among the pairs of rats involved and involved a sufficient number of pairs to be significant statistically, i.e., the difference between Diet A and Diet B in the female rats, averaging 20 gm ($P=0.016$) (Student, '25).

TABLE 3

Average body weights of paired rats at death of first rat in trio.

NO. CASES	AVERAGE AGE AT DEATH OF FIRST RAT IN TRIO DAYS	AVERAGE BODY WEIGHTS			NO. CASES	AVERAGE AGE AT DEATH OF FIRST RAT IN TRIO DAYS	AVERAGE BODY WEIGHTS		
		Diet A	Diet B	Diet C			Diet A	Diet B	Diet C
MALES					FEMALES				
5	498	285	307	...	5	465	197	217	...
2	473	224	...	237	6	364	199	...	208
5	577	...	259	283	1	601	...	248	250

Longevity

In order to minimize the spread of respiratory infections among the experimental rats, those obviously infected were separated from the rest and were always handled and fed last each day. The causes of death were largely respiratory in nature.

The average age at death in each diet and sex group is given in table 4. The table also includes the coefficients of

TABLE 4

Average age at death in days with coefficients of variation.

SEX	DIET A	DIET B	DIET C	SEX	DIET A	DIET B	DIET C
<i>Averages</i>				<i>Coefficients of variation</i>			
Males	641	756	630	Males	32	29	30
Females	627	563	632	Females	20	36	31

three dietary regimes. The average data summarized in table 2 show that mature weights were attained in from 40 to 60 weeks. They also reveal no significant differences in rate of growth among the three diet groups within the two sexes. The males continued to increase slowly in body weight up to the one hundredth week to an average weight of 330 to 340 gm, presumably due to fat deposition, and then, as a group, decreased rather precipitously until death. The females maintained an average body weight of 200 to 240 gm from the

TABLE 2

Average body weights in grams at different periods in the experiment.

TIME INTERVAL AFTER:	DIET A	DIET B	DIET C	TIME INTERVAL AFTER:	DIET A	DIET B	DIET C
<i>weeks</i>	MALES			<i>weeks</i>	FEMALES		
10	197	202	199	10	158	163	160
20	246	254	249	20	188	188	186
30	282	288	279	30	199	201	193
40	278	293	280	40	205	207(11) ¹	203
50	287(11) ¹	296	290(10) ¹	50	195	204(11) ¹	194
60	311(9) ¹	315(11) ¹	287(10) ¹	60	208(11) ¹	215(7) ¹	203(10) ¹

¹The numbers in parentheses denote the number of rats surviving at the indicated time when the original group size of 12 has been reduced by mortality.

thirtieth to the ninetieth week of the experiment, before the pre-mortal decrease in average weight supervened. The females on Diets B and C exhibited a greater average tendency to fatten in adult life than the females on Diet A, but the significance of these changes in group average weights in later life is obscured by the decreasing size of groups due to increasing mortality.

In table 3 are given the average body weights of the two surviving members of the trios in both sexes at the time of death of the first member of the trio. In ten of the twenty-four trios, the first death occurred in the rat subsisting on Diet C; in eight trios, the first death occurred in the rat on Diet B; and in six it occurred in the rat on Diet A. The average body

Among the female diet groups, none of the differences approached statistical significance. Among the male diet groups, the rats subsisting on Diet B stood out among the other groups as the longer lived, on the average, but the probabilities of 0.063 and 0.066 are still too large to be neglected. The suggestion contained in these low probabilities, that Diet B may have favored the greatest length of life in the male rats, must be tempered by the fact that, among the female rats, Diet B induced the shortest average life span of all three diets.

If the data are analyzed as unpaired groups according to Fisher's ('44, p. 122) modification of Student's method, somewhat larger probabilities of a chance outcome are secured among the male groups, and equal, or somewhat smaller probabilities among the female groups. The method of food intake control practiced in this experiment has, thus, not contributed appreciably to the effectiveness of the experiment.

The calcium content of the tissues

It is not believed that the calcium content of the fresh carcass or of the fresh tissues in experimental animals after natural death is of great significance, because of the probable disturbances in water balance associated with the terminal disease. Hence, all calcium analyses are expressed on the dry weight of tissue, or on the tissue ash. Even with these values there is no assurance that the terminal illness, with its inevitable disturbance in appetite, may not have modified the results.

²Table 6 summarizes the average per cent ash in the dry defatted bones (leg bones) and the average per cent calcium in the bone ash and the carcass ash. The differences between comparable group averages are small and are all statistically insignificant.

The average calcium contents of the samples of dry liver, kidney, heart and muscle are to be found in table 7. The averages for comparable groups suggest a tendency for the excessive-calcium group (Diet C) to possess soft tissues con-

TABLE 6

Average mineral content of the bones and carcass ash.

NUMBER OF CASES	SEX	DIET A	DIET B	DIET C
		%	%	%
Ash in dry defatted bones				
7 to 8	m	60.7	60.6	61.8
5 or 6	f	61.9	60.2	61.4
13 or 14	both	61.2	60.4	61.7
Calcium in bone ash				
7	m	36.1	37.6	37.2
4	f	37.8	38.0	37.6
	both	36.7	37.7	37.4
Calcium in carcass ash				
6 or 7	m	27.9	28.3	28.6
4	f	29.0	27.6	29.0
	both	28.4	28.1	28.8

TABLE 7

Average per cent calcium in the soft tissues expressed on the dry basis.

TISSUE	SEX	NUMBER OF CASES	DIET A	DIET B	DIET C
Liver	m	6 to 9	0.038	0.040	0.042
Liver	f	3 or 5	0.040	0.040	0.045
Liver	both	11 or 13	0.039	0.040	0.043
Kidney	m	6 to 9	0.065	0.062	0.090
Kidney	f	4 or 5	0.090	0.064	0.080
Kidney	both	11 to 13	0.073	0.062	0.085
Heart	m	6 to 9	0.048	0.048	0.076
Heart	f	3 or 4	0.097	0.040	0.050
Heart	both	9 to 13	0.063	0.045	0.068
Muscle	m	5 to 8	0.041	0.032	0.050
Muscle	f	4 or 5	0.034	0.042	0.052
Muscle	both	10 or 12	0.039	0.036	0.051
All tissues	both	0.054	0.046	0.062

taining the greatest degree of calcification. When the individual trios are examined, this tendency is almost completely obscured by the intra-trio variation in the case of the kidney and the liver. It is still quite evident for the heart and especially the muscle samples, however, and in the latter case the tendency is quite significant as between Diets C and B ($P=0.025$), but merely suggestive as between Diets C and A ($P=0.10$).

The individual data on the calcium content of the soft tissues, expressed on the dry basis, reveal no correlation with age at death. However, only three of the rats whose tissues were submitted to analysis died at ages less than 1 year, i.e., 307, 325 and 359 days. Also, no evident correlation exists among the calcium contents of the dry soft tissues for the same rat, indicating that a relatively high content of calcium in the dry liver, for example, gives no assurance that the other soft tissues in the same carcass will also show relatively high contents of calcium. This situation would indicate that calcium metastasis is primarily the result of local conditions in the soft tissues, rather than of systemic conditions.

SUMMARY AND CONCLUSIONS

An experiment on twenty-four trios of rats was undertaken to determine the effect of excessive calcium nutrition, especially in adult life, on growth, longevity and calcification of the tissues, such as occurs in many of the soft tissues during senescence. Twenty-four rats were carried from weaning to death on a diet containing about 1% of calcium. Another group of the same size subsisted on a diet containing 1% of calcium during the growing period and then only 0.7% for the remainder of their lives. A third group was raised on a 0.6% calcium level and then on a 0.22% level. The diets were otherwise satisfactory especially with reference to vitamin D and phosphorus.

The rate of growth was not appreciably affected by the different dietary regimes, though among the female rats the more liberal calcium diet was associated with a greater degree

TABLE 6

Average mineral content of the bones and carcass ash.

NUMBER OF CASES	SEX	DIET A	DIET B	DIET C
		%	%	%
Ash in dry defatted bones				
7 to 8	m	60.7	60.6	61.8
5 or 6	f	61.9	60.2	61.4
13 or 14	both	61.2	60.4	61.7
Calcium in bone ash				
7	m	36.1	37.6	37.2
4	f	37.8	38.0	37.6
	both	36.7	37.7	37.4
Calcium in carcass ash				
6 or 7	m	27.9	28.3	28.6
4	f	29.0	27.6	29.0
	both	28.4	28.1	28.8

TABLE 7

Average per cent calcium in the soft tissues expressed on the dry basis.

TISSUE	SEX	NUMBER OF CASES	DIET A	DIET B	DIET C
Liver	m	6 to 9	0.038	0.040	0.042
Liver	f	3 or 5	0.040	0.040	0.045
Liver	both	11 or 13	0.039	0.040	0.043
Kidney	m	6 to 9	0.065	0.062	0.060
Kidney	f	4 or 5	0.090	0.064	0.060
Kidney	both	11 to 13	0.073	0.062	0.065
Heart	m	6 to 9	0.048	0.048	0.076
Heart	f	3 or 4	0.097	0.040	0.050
Heart	both	9 to 13	0.063	0.045	0.065
Muscle	m	5 to 8	0.041	0.032	0.050
Muscle	f	4 or 5	0.034	0.042	0.052
Muscle	both	10 or 12	0.039	0.036	0.051
All tissues	both	0.054	0.046	0.062

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THE RETENTION OF NUTRIENTS IN CHEESE MAKING

IV. THIAMINE IN CHEDDAR CHEESE MADE FROM RAW AND PASTEURIZED MILK

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ONE FIGURE

(Received for publication April 1, 1946)

Studies of the fate of calcium, phosphorus and riboflavin in the manufacture of cheese have been reported recently (Irvine et al., '45a, b, c). The present report deals with the recovery of thiamine in Cheddar cheese made from raw and pasteurized milk, and its retention during ripening.

There have been relatively few values for the thiamine content of cheese reported in the literature, and the numbers of samples concerned have been very small. Booher and Hartzler ('39) reported a value of 42 μg per 100 gm in one sample of American Cheddar cheese assayed biologically. In studies on various types of cheese by a thiochrome procedure, Pyke ('39) found 12 μg of thiamine in 100 gm of Cheddar. Lane et al. ('42) and Cheldelin and Williams ('42) reported on American cheese. The former group, using a thiochrome method, found 42 μg in 100 gm of the edible portion and the latter workers found the thiamine content of one sample, as determined by the yeast-growth method, to be 26 μg per 100 gm of fresh cheese. Sullivan et al. ('43) did not include thiamine in their extensive study of water-soluble vitamins in various types of cheese.

Until recently there was no published information on the fate of thiamine during the manufacture and ripening of cheese. While the present study was in progress, Dearden et al. ('45) reported on extensive studies of several vitamins, including thiamine, in the production of Cheddar, Cheshire and Stilton cheese from unpasteurized milk. Approximately 15% of the thiamine present in the milk was retained in the cheese, and no significant losses occurred during ripening for periods up to 42 weeks. The ranges of thiamine content in ripe Cheddar, Cheshire and Stilton were, respectively, 53 to 57, 43 to 46 and 70 to 80 μg per 100 gm.

Numerous observations on the effect of pasteurization on the thiamine content of milk have been reported, and there is considerable variation in the destruction of thiamine found in different studies. Henry and Kon ('38) and Houston et al. ('40) reported that commercial sterilization causes losses of, respectively, 30% up to 50%, and the latter group found 10% destruction of thiamine in commercial pasteurization. Elvehjem ('41) reported a loss of 25%. More recently, the holder process of pasteurization was found to destroy 9% (Holmes et al., '43) and the high-temperature-short-time pasteurization to destroy 3% of the thiamine (Holmes et al., '45). Kendall ('42) observed 10 to 20% loss in pasteurization by unspecified methods. Because of these variable results and also because of the current interest in the question of pasteurization of milk for cheese manufacture, it was thought advisable to include this aspect in the present studies.

EXPERIMENTAL

The experimental lots of cheese were manufactured on four occasions at intervals of approximately 2 months. On each occasion, a bulk lot of 1600 to 1700 pounds of fresh milk from the College herd of Holsteins, Ayrshires and Jerseys, was thoroughly mixed and divided into three approximately equal weighed portions. The first portion was used in the raw state, the second was pasteurized by the holder method at 143°F. for 30 minutes, and the third portion was pasteur-

ized by the "High-Short" method at 161°F. for 16 seconds. Details of the pasteurization methods and the methods of cheese manufacture have been presented previously (Irvine et al., '45a, b). Samples of milk plus starter were taken from each of the vats before renneting, and samples of the first whey were also taken. The weight of cheese produced and the weight of the first whey were recorded. The press whey was not considered.

The cheese from each vat was divided into two lots, one of which was ripened at 40°F. and the other at 58°F. Samples were taken for analysis at 1 day, 14 days, and 1, 3, 6, 9 and 12 months. At 1 and 14 days, only one sample was taken for each vat, but at the other periods one sample was taken from each of the 40° and 50° ripening lots. The method of sampling has been outlined previously (Irvine et al., '45a).

METHODS OF ANALYSIS

The milk, whey and cheese samples were analyzed for total solids and thiamine. For the former the samples were prepared by thorough grinding in a kitchen food grinder. Moisture loss from weighed portions in aluminum moisture dishes was determined after 24 hours at 100°C. Since the thiamine analyses were carried out with some slight variations in technical details from standard methods, our technic is described in detail.

Method for thiamine

The samples of cheese for thiamine determination were finely grated through a bronze screen of 20-mesh. This resulted in a rather feathery material for extraction. After thorough mixing, 20 gm of this material was weighed into a 125-ml Erlenmeyer flask, and to this were added 75 ml N/10 H₂SO₄ and 6 ml of 2.5 M sodium acetate solution. The resultant pH of this mixture was approximately 4.8. Five ml portions of freshly prepared solutions of papain and of takadiastase were then added. These solutions contained 0.4 gm of enzyme in 5 ml, the takadiastase solution being pre-

The thiamine values for the milk and for the various lots of cheese at different stages of ripening are presented in table 2. The values for the milk suggest that very little, if any, destruction of thiamine occurred in either pasteurization process.

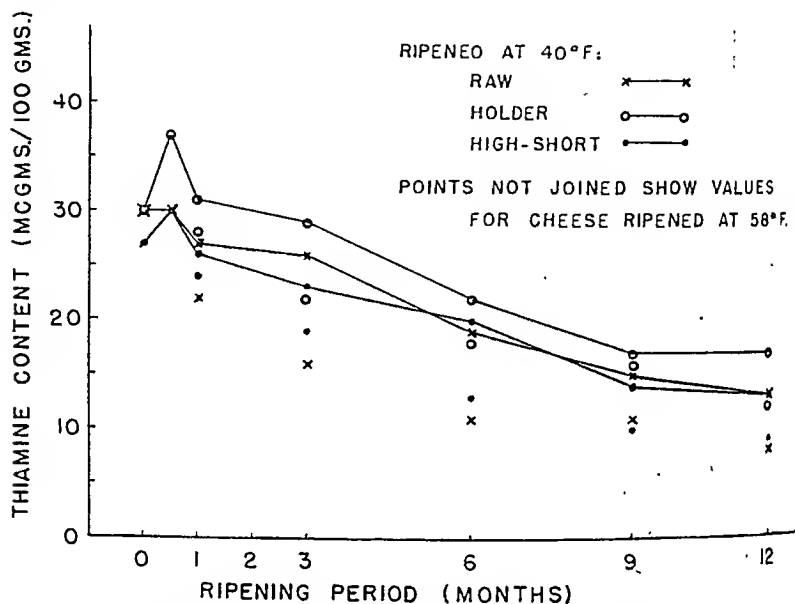


Fig. 1 Graph showing decrease in thiamine content of Cheddar cheese during ripening. The values shown are the averages for four batches and have been adjusted to the basis of 35% moisture content.

The thiamine content of the cheese samples showed a progressive decrease during ripening. This was true at both ripening temperatures, but invariably the thiamine loss was greater in the cheese ripened at 58°F. The average losses in 12 months ranged from 43 to 52% for 40° ripening³ and from 60 to 73% for ripening at 58°F. These progressive losses are readily seen from the curves in figure 1 in which have

³ A recent regulation of the Canadian Government forbids the sale of pressed cheese which has been ripened at a temperature below 45°F. This and similar regulations in force in many states of the United States are designed to eliminate cheese as a carrier of typhoid fever organisms. The present work, initiation of which antedated the Canadian regulations, should not be construed as an argument in favor of 40°F. ripening at the risk of typhoid contamination.

been plotted the averages of the four batches of cheese. It will be noted that the thiamine levels are highest throughout ripening in the cheese manufactured from milk pasteurized by the "Holder" process, the situation being the same for both ripening temperatures. This relationship is substantiated by the fact that graphs plotted for the individual batches, but not presented herewith, show this same behavior in all cases with but one possible exception. In these individual graphs, too, it is observed that the thiamine levels for the "raw" and "high-short" samples are, in general, very close together, appreciably lower than those of the "Holder" samples. These facts suggest that the thiamine decrease on ripening may be influenced by some catalytic factor which is inactivated at least partially by the "Holder" method of pasteurization but not by "High-Short" pasteurization. The authors can suggest no satisfactory explanation for the apparent increase at 14 days which is shown by the composite figure 1 and by the data for many of the individual batches.

SUMMARY

The fate of thiamine in the manufacture of Cheddar cheese and the effect of ripening periods up to 12 months upon this vitamin in the cheese produced have been studied. Cheese was manufactured at four 2-month intervals, covering the spring, summer and fall seasons. On each occasion, raw milk and milk pasteurized by two different methods were used for the cheese manufacture.

Milk pasteurized by either the "Holder" or the "High-Short" process showed little or no loss of thiamine.

The process of cheese manufacture caused no actual destruction of thiamine. The average retention in the cheese of this vitamin present in the milk was 8.8%. This is higher than can be accounted for on the basis of water-solubility, indicating that some of the thiamine is "bound" to some portion of the milk solids, presumably protein.

A progressive decrease in thiamine content of the cheese with increasing ripening period was observed. The losses in

12 months ranged from 43% to 73%. Ripening at 58°F. caused greater decreases than ripening at 40°F.

Cheese manufactured from milk pasteurized by the "Holder" process showed higher thiamine levels throughout ripening than the cheese from raw or "High-Short" pasteurized milk.

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REPRODUCTIVITY AND GROWTH OF ALBINO RATS ON A PROLONGED DAILY INTAKE OF CAFFEINE

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ONE FIGURE

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Studies made by different investigators on the effect of caffeine on reproductivity and growth have led to divergent conclusions. Stieve ('30, '31) described degenerative changes in the testicles of rabbits that had been given caffeine in a coffee brew by stomach tube for 3 to 4 weeks. These changes he believes accounted for the smaller number of offspring delivered by the females in his test group on coffee as compared with the controls given an equal amount of Kaffee Hag containing only a minute amount of caffeine. Vacca ('26) had previously reported a deleterious action of caffeine on the dog's testicle. Eichler and Mügge ('32), however, found that daily subcutaneous injections of caffeine did not result in a diminution of the reproductive capacity of albino rats over four generations. Their observations did not extend over the entire reproductive period of any one of the four generations. Hammill and Miller ('35) reported that litters of their test animals given caffeine containing beverages were of normal size. At variance with these results are the preliminary observations of Cheney ('44) indicating a reduced fecundity in rat colony studies extending over a period of 5 years.

As regards growth, some investigators (Hammill and Miller, '35; Smith and Hamburger, '36) report that prolonged

administration of caffeine retards it. Others (Stieve, '30; Eichler and Mügge, '32) found that the drug had no effect. In the experiments of Scott and Chen ('44) there was no depression in growth when the food mixture given the animals contained caffeine in a concentration as high as 0.1%.

The indecisiveness of these studies suggested the advisability of the present investigation. Because of the extensive use of the caffeine-containing beverages, coffee and tea, the problem assumes practical interest from the nutritional point of view.

PROCEDURE

Twenty-five groups of albino rats were set apart at weaning for the experiment. Each group consisted of two males and two females from the same litter. One pair (male and female) served as test animals, the other pair as controls. All the animals were fed the same ration of commercial dog food¹ supplemented with green food and cod liver oil and were allowed to eat ad libitum. The drinking fluid to which there was free access at all times was tap water for the controls and a sweetened beverage containing caffeine for the test animals. This beverage constituted the sole fluid intake from the time of weaning to the conclusion of the experiment. It was chosen in preference to a solution of caffeine in water in order to insure as large an intake of caffeine as possible. Preliminary experimentation had revealed that the animals would drink a much larger quantity of this beverage than a solution containing the same amount of caffeine in plain water. The fluid was placed in an inverted graduated test tube provided with a drinking tip to which the animal had ready access. Various tests and observations had shown that there was no loss of fluid when the apparatus was left undisturbed and an extremely small loss when the animal was drinking. This was estimated to be considerably less than 5% of the contents of the container. The fluid intake of the adult control animals was approximately 30 ml per day

¹ Purina Dog Chow.

and that of the test animals 60 ml. This volume of the caffeine solution contained 12 mg caffeine which gave an average daily intake of approximately 40-50 mg per kilo body weight.

When the animals were 100 days old they were mated, a control male with a control female and a test male with a test female. Each pair was kept in a separate cage throughout the experiment. Records were kept on the date of birth of each litter, the number of offspring and the number of young that survived to weaning at the age of 21 days. The experiment was terminated for the individual pairs of animals when the females failed to become pregnant within 3 months after the birth of the last litter. The animals were then sacrificed and the testicles of the males removed and prepared for histological examination. The experiment was carried on continuously over 3½ years.

RESULTS

Reproductivity

The experimental data presented in table 1 show that there was considerable variation in the number of litters and number of offspring in each litter among both test and control animals. The averages were slightly higher in the test than in the control animals but the differences were not statistically significant. The critical ratio was only 1.3 times the difference.

The control animals gave birth to 105 litters containing a total of 618 offspring, or an average of 5.9 per litter; the test females delivered 135 litters containing 714 offspring, or an average of 5.3 per litter. Two of the control females produced no offspring whereas all of the females of the test group had at least one litter. There were four females in the control group and one in the test group that failed to raise a single animal to the weaning stage. 52.2% of the total number of offspring of the test animals and 53.2% of the controls survived to weaning. The average length of time between mating and delivery of the first litter was 31 days for the controls and 29 days for the test animals.

TABLE 1

Reproductivity of albino rats on a caffeine-containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

TESTS				CONTROLS		
Group number	Number of litters	Number of offspring	Number weaned	Number of litters	Number of offspring	Number weaned
1	3	29	13	1	5	0
2	5	16	0	6	40	32
3	11	71	47	6	34	22
4	4	32	20	14	74	33
5	4	28	9	1	3	0
6	4	14	1	0	0	0
7	9	32	7	6	39	18
8	11	45	33	6	45	39
9	7	50	19	3	17	8
10	9	58	37	13	76	61
11	1	8	4	2	10	6
12	4	27	22	2	8	3
13	4	23	16	3	24	9
14	1	5	5	7	44	3
15	10	42	27	4	18	16
16	4	18	4	2	9	0
17	7	32	12	6	34	24
18	3	13	9	1	8	0
19	1	8	5	2	19	5
20	6	27	17	3	18	11
21	9	30	11	3	19	5
22	5	27	10	1	9	2
23	7	52	27	7	29	12
24	3	15	10	0	0	0
25	3	12	8	6	36	20
Total	135	714	373	105	618	329
Average per group	5.4	29	15	4.2	25	13

Testicular histology

A histological examination² was made on the testicles of twenty-three of the test males and fifteen controls. The find-

²All histological studies were done by Dr. Walter Sheldon, pathologist at Grady Hospital, Atlanta, Ga.

ings on 57% of the tests and 53% of the controls were essentially negative. In the remainder there occurred certain histological changes which, however, were of the same type in both the tests and controls. None of the lesions resembled those described by Stieve in rabbit's testicles. In some of the testicles small patchy areas were observed in which the seminiferous tubules had become atrophic. These tubules were lined by a thin, irregular layer of atrophic epithelium suggesting fatty degeneration. There was no trace of spermatogenesis left. In these areas there was some increase in the interstitial cells but no appreciable thickening of the tubular basement membrane. The remaining portions of the testes were normal with normal spermatogenesis. In other testicles there was a slight general reduction in spermatogenesis but no areas of atrophy. Several showed extensive areas of tubular atrophy and fibrosis with reduced spermatogenesis in the remaining tubules. These changes may be accounted for by spontaneous physiological involution.

No relationship could be detected between the histological changes in the testicles and the number of offspring. For example, in animal no. 17 of the test group, the testicles showed fairly extensive lesions and in animal no. 20 the lesions were very slight. However, the former sired seven litters containing a total of thirty-two young, while the latter sired only six litters containing twenty-seven young. In both these instances the number of offspring was larger than in many cases in which no histological changes were seen in the testicles.

Growth

Weekly weighings were made on ten test and ten control males for 26 weeks and on an equal number of females for 11 weeks. The weighings were discontinued on the females at this time because they were then mated and became pregnant shortly thereafter. From the weight curves presented in figure 1 it is apparent that the daily ingestion of caffeine had no retarding effect on growth.

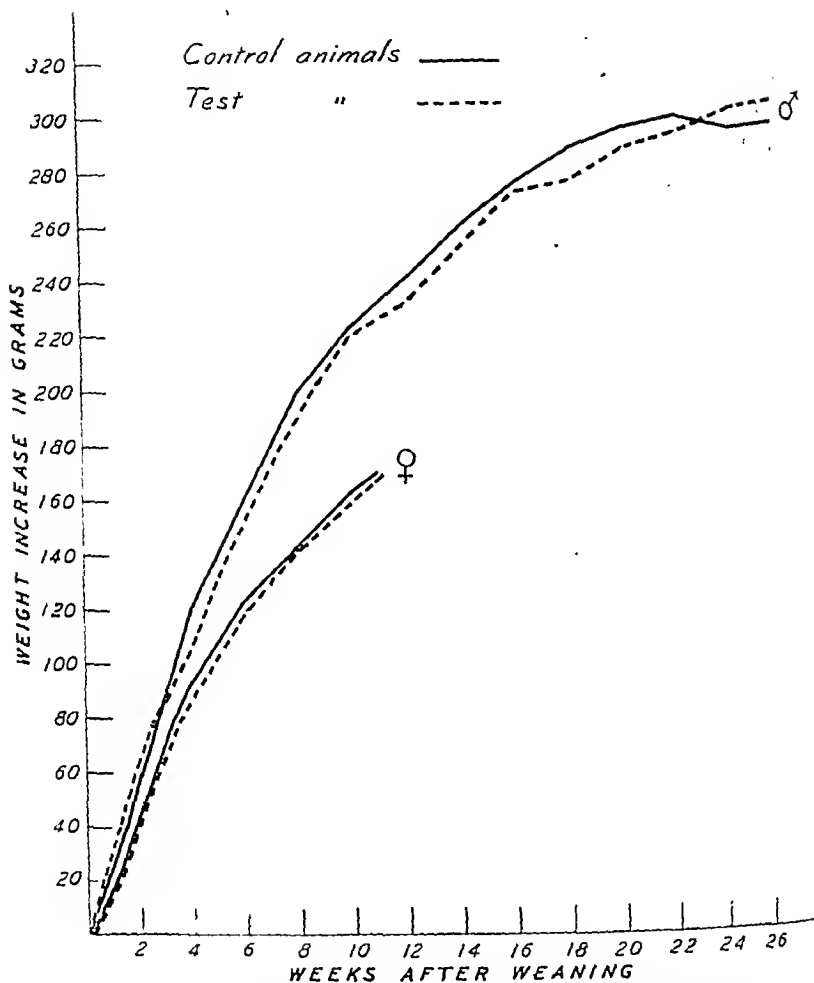


Fig. 1 Growth curves of albino rats on a caffeine containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

DISCUSSION

In the experiments of Eichler and Mügge ('32) it was found that the prolonged administration of caffeine to albino rats produced no diminution in reproductive capacity over four generations. Experimentation on each generation was terminated after the birth of the first litters. From our observa-

tions on both the number of offspring and the testicular tissue of the male parents, it is apparent that throughout the reproductive period of the animals' life there was no evidence of sterility as a result of the daily ingestion of relatively large amounts of caffeine.

The discrepancy between our observations and those of Stieve ('30, '31) on the testicle of the rabbit and those of Vacca ('26) on the dog's testicle might conceivably be due to a species difference in response to caffeine. This explanation, however, can not be accepted without further experimentation. The experiments of Stieve, as well as those of Vacca, appear to have been poorly controlled. The only information available with regard to Stieve's animals is that they were pure blooded Russian rabbits, 7 to 16 months old with an average weight of 1865 gm for the females and 1887 gm for the males. In studies of this kind it is of paramount importance that environmental and nutritional conditions be the same for both the test and control animals before and during the experiment, for it has been shown that such factors as light (Fiske, '39, '41; Pomerat, '42) and vitamin deficiencies and inanition (Mason, '30, '33; Mason and Wolfe, '30; Trusecott, '44) affect the pituitary which, as is well known, exercises through its gonadotropic hormone a direct action on the testicles. In Vacca's experiments ('26) not only were the four dogs used as test animals selected at random but also the amount of caffeine administered was obviously toxic. All the test animals became emaciated and had general tremors and convulsions.

The difference in our results as compared with those obtained by Hammill and Miller ('35) with respect to growth may be due to the fact that in their experiments the various groups of animals were not fed the same diet. The authors state that all the diets, the composition of which is not given, were satisfactory for experimental purposes, but it is possible that they might not have been equally effective in promoting growth.

This objection does not apply to the observations of Smith and Hambourger ('36). However, it should be noted that their animals were given 100 mg caffeine per kilo by stomach tube whereas ours ingested ad libitum the smaller amount of 40 to 50 mg per day. It is hoped that further experimentation now under way in our laboratory may show whether the differences between our results and theirs were due to the different amount of caffeine the animals received or the different experimental procedure.

SUMMARY

Albino rats were given a sweetened caffeine beverage as their sole source of fluid throughout their reproductive period.

The average daily consumption of caffeine by each animal amounted to 40-50 mg per kilo body weight.

The reproductive capacity as judged by the number of litters and number of offspring was not impaired by the daily ingestion of caffeine.

There were no pathological changes in the testicles of the test animals except for the natural histological degenerative changes which were observed in about equal frequency in the control animals.

The rate of growth of the animals on the caffeine beverage was the same as that of the controls.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

VIII. THE UTILIZATION OF THIAMINE DURING LACTATION ¹

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EIGHT FIGURES

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The metabolism of thiamine during the reproductive cycle, especially during lactation, is largely a matter of conjecture. In recommending dietary allowances for lactating women, the Food and Nutrition Board of the National Research Council ('45), after reviewing the published data dealing with thiamine requirement, suggested that 2.0 mg of thiamine be allowed for a diet containing 3000 cal. This thiamine to calorie ratio of 0.67 is somewhat higher than the 0.5 mg thiamine per 1000 cal.³ recommended for normal adults. It was increased because additional thiamine may be needed for the synthesis of milk, to give added protection during the child-bearing period. Some believe that the allowance has been placed too high, but as the Food and Nutrition Board pointed out, the recommendations do not constitute requirements, but were selected to provide margins of safety. Variations in requirements are known to be large owing to differences in size and

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³ Kilogram calories.

absorption as well as efficiency of utilization, and the margins of safety are for those whose requirements are above the average.

There is evidence that the non-fat calories of a diet may be a better basis for estimating thiamine requirement than the total calories. Reinhold, Nicholson and Elsom ('44) found that increased amounts of thiamine were utilized when the proportion of carbohydrates in the diet was high. The so-called "sparing action" of fat appears to be related to a coincident decrease in the carbohydrate content of a diet. The decreased thiamine excretion on a high carbohydrate diet is interpreted as indicating an increased need for thiamine in the metabolism of the carbohydrate; therefore, the proportion of carbohydrate in the diet may be an important factor in determining the daily need for thiamine. Perhaps weight or surface area should also be considered. Elsom, Reinhold, Nicholson and Chornock ('42) in studies of the B vitamins fed six human subjects thiamine in amounts consistent with the theoretical requirement based on Cowgill's ('34) formula and found that three subjects developed deficiency symptoms. These were the three smallest people in the group and this prompted Elsom to conclude that smaller people eating less food and therefore receiving fewer calories may have higher requirements per unit of body weight than larger people. Recent work of Ayre and Bauld ('46) has emphasized the importance of adequate thiamine intake: "The finding of abnormal estrogenic activity coupled with thiamine deficiency in cases of menorrhagia and uterine cancer suggests a possible etiological correlation between the dietary deficiency, the abnormal estrogen level, and the pathological lesion."

EXPERIMENTAL PROCEDURE

By chemical analysis, the thiamine intakes of nursing mothers at various stages of lactation have been determined, as well as their daily thiamine excretions in urine and secretions in breast milk during the same periods. No attempt was made to collect feces since, although it is known that

bacteria synthesize the B vitamins in the intestinal tract, it is not known whether this material is available to the human organism. Most of the experimental evidence indicates that (a) fecal thiamine is unrelated to the intake (Leong, '37), except in cases of very large intakes, (b) it is chiefly present in the bacterial cells and unavailable to the animal organism (Abdel-Salaam and Leong, '38; Emerson and Obermeyer, '45), and therefore, it is probably insignificant in studies on normal diets.

The subjects of the investigation were multiparas with medical records of good or excellent health and having successfully nursed their other children. Of the fourteen women participating in the study seven were studied during two consecutive 5-day periods immediately following delivery. Five were observed for 1 day, 7 to 9 days postpartum. Ten of the fourteen mothers were studied during eighteen 5-day periods at various intervals during the production of mature milk. All of the diets were comparable, qualitatively, but the amounts of all foods except milk were increased or decreased so that the appetites of the women were satisfied. Duplicates of each meal were added to composites which were analyzed for each 5-day period. Complete 24-hour collections of milk and urine were obtained. Details of selection of subjects, and the methods of manually expressing the milk and of collecting the food and milk are given in preceding publications (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Davies, '45). In conjunction with many periods during which mature milk was collected, fasting samples of urine were obtained the first morning of the 5-day period and the morning following the period. Each collection represented excretion for 1 hour, 12 to 13 hours following the last meal. Subsequent papers will present the data for other vitamins and minerals.

Sample preparation and analysis

Food. Approximately 400 gm of the ground, 5-day food composite was homogenized in a Waring Blendor and the

volume made to 500 ml. Of this, 20 ml were used for each of duplicate determinations. Each sample was diluted to 75 ml with water and enough 1 *N* sulfuric acid to make the mixture 0.1 *N* and autoclaved for 10 minutes at 15 pounds pressure. When cool, the solution was adjusted to pH 4.5 with 2.5 *M* sodium acetate, then incubated under toluene with clarase overnight (about 16 hours) at 37°C. Finally, the sample was diluted to 100 ml, filtered, and 40 ml of the filtrate used for the thiamine determination. Riboflavin was determined in the remaining filtrate (Roderuck, Coryell, Williams and Macy, '45).

Milk. An aliquot of milk from each expression during a 24-hour period was poured immediately into a jar which was kept tightly covered in a refrigerator. The composite for the day preceding was taken to the laboratory each morning and held under refrigeration pending analysis by the method of Hennessy ('42) slightly modified (Roderuck, Williams and Macy, '45). The majority of the assays were made within 24 hours.

Urine. Each voiding of urine was added to an amber glass bottle containing acetic acid and toluene. The 24-hour composite was 2% acetic acid. Each day the composite was taken to the laboratory, the volume recorded and aliquots removed for analysis by the thiochrome procedure (Roderuck, Coryell, Williams and Macy, '45). Filtration was the only pretreatment required with urine, since all the thiamine was in the free state, so measured amounts of filtered urine were added directly to Decalso column. Usually 10 ml per liter of urine excreted was used for each determination. The sulfite treatment described by Mason and Williams ('42) was satisfactory in obtaining blank determinations when 50 mg of sodium sulfite was used for each sample, instead of the 25 mg which they recommended.

RESULTS AND DISCUSSION

Table 1 gives for all the women studied during the first 10 days postpartum the daily volumes of milk and urine,

the average daily thiamine intakes, the daily secretions of free and total thiamine in milk, and the daily excretion of thiamine in the urine. Averages of all values obtained for each of the 10 days are given in table 2. While only insignificant amounts of thiamine were secreted in the colostrum during the first 2 days postpartum, the rapid increases in immature milk volume, with greater concentration (Roderuck, Williams and Macy, '45), resulted in an average daily secretion of 95 μ g on the tenth day. Although larger amounts of free thiamine also were secreted, the major portion of the increases in the amounts of total thiamine in milk during the puerperium consisted of bound thiamine. A preceding paper (Roderuck, Williams and Macy, '45) pointed out that the concentration of total thiamine in colostrum during the first 4 days of lactation varied from 0.9 to 2.4 μ g, increasing to an average of 8.1 μ g per 100 ml on the tenth day. The average concentration of free thiamine was not more than 0.5 μ g the first 6 days of lactation and the highest value was 1.0 μ g on the ninth day postpartum.

Within the range of thiamine intakes in this study there was no relationship between thiamine intake and secretion in milk. The scatter diagrams in figures 1 and 3 demonstrate the increases in milk volume and secretion of thiamine in milk during the first 10 days postpartum. The data in tables 1 and 2 and the values plotted in figures 1 to 8 inclusive show no relationship among thiamine intakes, urine volumes and levels of thiamine excretion in urine. The values for the different women (table 1) show parallel trends in increasing milk volume and thiamine secretion in milk during the first 10 days postpartum.

The average values obtained for each 5-day period during the puerperium and during mature milk production are given in table 3. The daily milk and urine volumes and the percentages of the average thiamine intake secreted in milk and excreted in urine are plotted in the figures 1 to 8. Although evidence obtained with rats has shown that additional thiamine is required to support satisfactory milk production

TABLE 1

Thiamine intakes, excretion in urine and secretion in immature milk during first 10 days postpartum.¹

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	ml	ml	mg	mg	mg	mg
L.F.	1	71	2590	1.10	1	0	315
	2	200	1275	1.10	2	2	215
	3	733	2420	1.10	9	2	285
	4	1122	2075	1.10	19	6	210
	5	1441	1674	1.10	25	9	225
	6	1501	1718	1.12	35	10	192
	7	1596	1340	1.12	49	11	150
	8	1638	1810	1.12	64	13	149
	9	1676	1374	1.12	105	21	138
	10	1872	2220	1.12	129	20	172
V.G.	1	82	2067	0.82	2	..	104
	2	177	1689	0.82	3	1	107
	3	849	1775	0.82	16	3	113
	4	1413	1323	0.82	25	1	108
	5	1471	1303	0.82	27	2	103
	6	1782	1148	0.73	47	4	93
	7	1630	1230	0.73	59	3	109
	8	1895	1237	0.73	97	7	95
	9	1828	1104	0.73	106	12	119
	10	1770	1075	0.73	120	13	95
V.K.	1	9	3086	0.92	146
	2	90	3651	0.92	1	..	187
	3	484	2596	0.92	6	..	126
	4	547	2427	0.92	10	..	141
	5	560	2331	0.92	15	..	134
	6	663	2249	0.98	23	3	164
	7	781	3380	0.98	40	4	183
	8	775	3840	0.98	51	6	156
	9	794	3115	0.98	59	8	151
	10	797	3328	0.98	65	7	143
V.L.	1	30	1757	1.25	196
	2	56	2686	1.25	183
	3	353	2759	1.25	6	1	171
	4	794	1823	1.25	14	2	170
	5	844	1847	1.25	18	3	137
	6	955	2710	1.34	33	4	196
	7	1047	2841	1.34	47	3	255
	8	1098	2066	1.34	59	12	209
	9	1118	2151	1.34	75	7	174
	10	1200	1669	1.34	80	10	162

TABLE 1—(continued)

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	ml	ml	mg	μg	μg	μg
J.M.	1	35	2182	1.15			60
	2	385	1386	1.15	6	1	108
	3	870	1519	1.15	14	2	94
	4	1011	1201	1.15	24	3	94
	5	1121	1283	1.15	38	4	90
	6	1125	1172	1.25	51	6	101
	7	1287	1161	1.25	76	7	163
	8	1136	1520	1.25	89	10	141
	9	1258		1.25	112	11	
	10	1336	1553	1.25	136	12	148
C.O.	1	16	1864	1.00			67
	2	100	939	1.00	1		62
	3	335	1511	1.00	4	0	87
	4	595			7	3	
	5	725	..		13	4	
	6	821		1.05	11	4	
	7	798	1470	1.05	24	6	131
	8	950	1127	1.05	39	5	87
	9	931	1191	1.05	42	9	104
	10	660		1.05	43	11	.
V.S.	1	6	1869	1.31			513
	2	92	1874	1.31	2		444
	3	420	2387	1.31	9	1	390
	4	600	2063	1.31	14	2	288
	5	697	2065	1.31	22	2	261
	6	756	1502	1.32	37	5	249
	7	818	2524	1.32	51	6	352
	8	837	2237	1.32	64	10	296
	9	932	1984	1.32	82	10	284
	10	924	2287	1.32	92	11	264
M.B.	9	1189	1035	1.28	125	23	47
E.L.	8	688	677	1.06	22	6	72
M.S.	7	880	1355	0.88	43	10	81
G.S.	7	1017	1981	1.04	40	5	146
F.W.	7	953	1032	1.23	44	7	74

¹ The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F., 23.5 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L., 5 hours; J.M. 16 hours; and V.S. 7 hours.

(Macy, Outhouse, Long and Graham, '27), in the present investigation diets which supplied amounts of thiamine much lower than the Recommended Dietary Allowances of the Food and Nutrition Board ('45) provided adequate amounts for the mothers' bodies and for milk production without depressing the rates or amounts of urine excretion. It should be

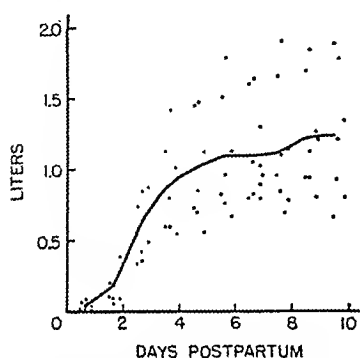


Fig. 1 Volume of immature milk secreted per 24 hours.

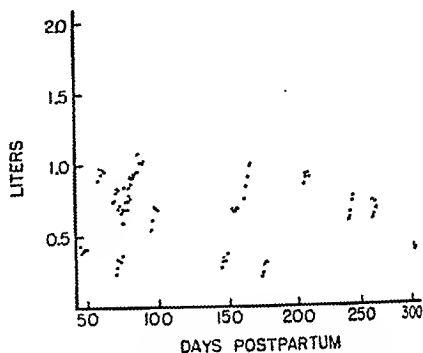


Fig. 2 Volume of mature milk secreted per 24 hours.

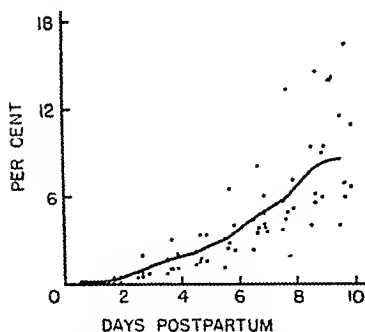


Fig. 3 Percentage of average thiamine intake during 5 days secreted into 24-hour collections of immature milk.

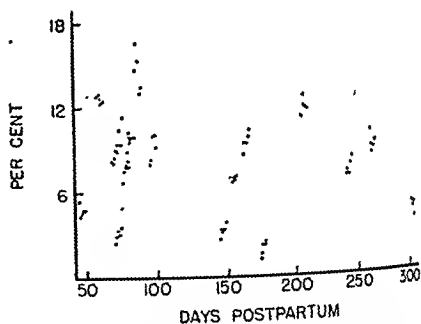


Fig. 4 Percentage of average thiamine intake during 5 days secreted into 24-hour collections of mature milk.

pointed out that the volume of milk during the last few days of the lying-in period is largest, but the concentration at that time is small, so that the total daily output is also small. The concentration of thiamine, as pointed out in an earlier paper (Roderuck, Williams and Macy, '45), remained rela-

tively constant from the fifth week of lactation until milk flow diminished. The average daily secretion in mature milk seems to have been related to milk volume rather than to thiamine intake. Even during the second to third month of

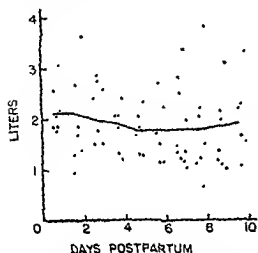


Fig. 5 Volume of urine per 24 hours during period of secretion of immature milk.

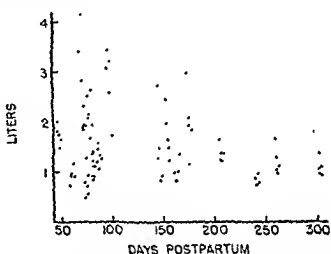


Fig. 6 Volume of urine per 24 hours during period of secretion of mature milk.

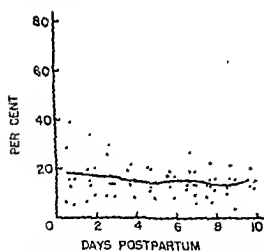


Fig. 7 Percentage of average thiamine intake during 5 days excreted in 24-hour collections of urine during period of secretion of immature milk.

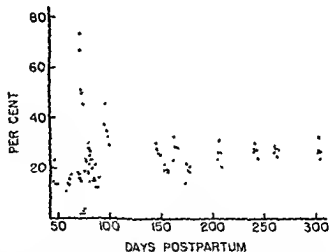


Fig. 8 Percentage of average thiamine intake during 5 days excreted in 24-hour collections of urine during period of secretion of mature milk.

lactation, which is the period of highest secretion of thiamine in the milk, all of the averages for thiamine output in milk were less than 15% of the intake (fig. 4). Perhaps, then, in lactation the thiamine requirement is increased only by this amount.

That the largest percentages of thiamine unaccounted for in milk and urine occurred in the 5-day period following parturition suggests that muscle metabolism during labor may have drawn extensively on the mothers' thiamine stores, producing a need for replenishment of their reserves. The thiamine concentration in blood has been shown to be lower after birth than at the end of pregnancy (Gaehtgens and Sauerbrey, '43; Neuweiler, '42). The average daily intake of thiamine was somewhat lower for this period than for any other period and therefore may have been utilized more efficiently.

TABLE 2

Thiamine in 24-hour collections of immature milk and urine during the first 10 days postpartum.

INTERVAL POSTPARTUM	MILK			URINE		
	Volume	Thiamine		Volume	Thiamine	
<i>days</i>	<i>ml</i>	<i>μg/day</i>	<i>μg/100 ml</i>	<i>ml</i>	<i>μg/day</i>	<i>μg/100 ml</i>
1	36	1	1.5	2202	200	9.6
2	157	3	1.4	1928	187	10.5
3	578	9	1.6	2210	181	7.8
4	869	16	1.8	1819	163	9.2
5	980	23	2.4	1750	158	9.0
6	1086	34	3.2	1750	166	9.8
7	1081	47	4.5	1831	164	9.2
8	1127	61	5.5	1814	151	8.9
9	1216	88	7.4	1708	145	8.7
10	1223	95	7.9	2022	165	8.6

During the sixth to tenth days, 13-15% of the thiamine appeared in the urine, approximately the same percentage as during the first 5 days postpartum, but the milk thiamine increased from 3.0 to 8.5% of the intake. In the periods of mature milk secretion the percentages of thiamine in milk increased only slightly, whereas the percentages of thiamine in the urine increased noticeably which may indicate greater saturation of the mothers' tissues as lactation progressed.

The average percentage of thiamine intake accounted for during the mature milk periods was 31%; of this, 23%, an average of 305 μg per day, appeared in the urine. According

TABLE 3

Average daily thiamine intakes, excretion in urine and secretion in milk during thirty-two 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE						
		Milk	Urine	Intake	Milk				Urine	
					Total	Free				
	days	ml	ml	mg	μg	% intake	μg	% intake	μg	% intake
V.G.	1-5	798	1631	0.82	14	1.7	2 ¹	0.2	107	13
	6-10	1781	1159	0.73	86	11.6	8	1.1	102	14
	78-82	848	1281	1.36	126	9.2	33	2.4	318	23
	161-165	901	993	1.18	112	9.5	90	7.6	331	28
	239-243	681	810	1.18	89	7.5	81	6.8	320	27
	302-306	394	1080	1.38	62	4.5	58	4.2	375	27
V.K.	1-5	338	2818	0.92	6 ¹	0.6			147	16
	6-10	762	3182	0.98	48	4.9	6	0.6	160	16
	95-99	647	2804	1.07	96	9.0	23 ¹	2.1	382	36
	144-148	325	1447	1.20	39	3.2	11	0.9	326	27
V.L.	1-5	415	2174	1.25					173	14
	6-10	1084	2287	1.34	59	4.4	7	0.5	199	15
	68-72	789	2849	1.50	127	8.5	25 ¹	1.7	243	16
	152-156	680	1756	1.61	111	6.9	40	2.5	315	20
J.M.	1-5	684	1514	1.15	16 ¹	1.4	3 ¹	0.3	89	8
	6-10	1228	1352	1.25	93	7.4	9	0.7	140 ¹	11
	75-79	708	2265	1.38	97	7.0	24 ¹	1.7	286	21
	173-177	268	2003	1.59	30	1.9	10	0.6	297	19
B.S.	85-89	1020	1347	1.11	162	14.6	40	3.6	169	15
	204-208	913	1356	1.32	158	12.0	75	5.7	333	25
	259-263	676	1219	1.26	115	9.2	63	5.0	336	27
V.S.	1-5	363	2152	1.31	9 ¹	0.7	2 ¹	0.2	379	29
	6-10	853	2107	1.32	65	4.9	8	0.6	289	22
	70-74	304	1852	1.40	43	3.1	24	1.7	798	57
L.F.	1-5	713	2007	1.10	11	1.0	4	0.4	250	23
	6-10	1657	1692	1.12	76	6.8	15	1.3	160	14
C.O.	1-5	354	1438	1.00	5 ¹	0.5	2 ¹	0.2	72 ¹	7
	6-10	832	1263	1.05	32	3.0	7	0.7	104 ¹	10
M.B.	72-76	718	691	1.06	106	10.0	41	3.9	27	2
M.M.	45-49	401	1741	1.36	65	4.8	35 ¹	2.6	217	16
M.S.	58-62	947	939	1.17	147	12.6	39	3.3	165	14
J.S.	80-84	899	1078	1.41	132	9.4	40	2.8	256	18

¹ Less than five 24-hour collections included in average. For total thiamine in milk values are not obtained for first day but values calculated from concentration in milk of second day are included in average (table 1).

to values in the literature for excretion by normal adults, the nutritional status of the mothers studied was satisfactory with respect to thiamine. The average values for urine in the two periods during the puerperium, 174 and 165 μg per day, respectively, also exceed the figures which are considered normal. Toverud ('39) found an average excretion of only 70 μg per 24 hours from lactating women who were receiving a diet calculated to provide 1.5 to 2.0 mg per day. Melnick, Field and Robinson ('39) found that normal women excreted about 90 μg per day, or 14% of their intake. This figure is lower than their average for normal males, who excreted 198 μg per day, 20% of their intake, and is considerably lower than the values found in this study. Jolliffe, Goodhart, Gennis and Cline ('39) found that 7 to 24% of the thiamine intake appeared in the urine. Normal individuals, according to Carden, Province and Ferrebee ('40), excrete somewhere between 10 and 40% of the thiamine in their diet. They found an average excretion of 182 mg per day for twelve normal adults. Williams, Mason and Wilder ('43) found that when the intake of thiamine was approximately 1 mg per day, an excretion of 100 μg or more in 24 hours represented normal excretion.

After a few weeks on a diet containing 0.33 mg thiamine per 1000 cal., the subjects of an experiment conducted by Keys, Henschel, Mickelsen and Brozek ('43) excreted as an average about 10% of their intake in their urine. On the basis of physical tests, clinical observations and subjective reports, this intake was considered to be more than adequate. In this study the average thiamine intake per day was slightly lower than the recommendation of the Food and Nutrition Board ('45) for women weighing 56 kg and considerably lower than the recommended allowance corrected to the weights of the women (Kancher, Moyer, Williams and Macy, '46), but the urine data presented indicate that these subjects were not thiamine deficient, although thiamine intakes were lower than the recommended allowances and the average thiamine to calorie ratio was 0.4 instead of 0.7. Holt

('44) believes that 0.24 to 0.44 mg thiamine per 1000 cal. is protective, and that the same values appear to be valid for all age groups and for pregnant women. His maximum value also seems adequate for lactating women in data presented here.

The diets during the study were high in fat and protein, therefore, one might expect the B₁ requirements to be somewhat lower than on a diet higher in carbohydrate.

Analyses showed 41% of the calories were contributed by fat, a percentage well above the 20 to 25% recommended by the Food and Nutrition Board. Non-fat calories were calculated from the percentage of fat in food composites which were analyzed after drying under vacuum from the frozen state (Flosdorf and Mudd, '38). The values were converted to calories with the factor 9.45. The non-fat calories for the mature milk periods averaged 59% of the total calorie intake. The average ratio of thiamine in the milk to non-fat calories in the diet was 0.76.

Fasting levels of thiamine in urine have been suggested by some investigators as a reliable index of thiamine status. Johnson, Sargent, Robinson and Consolazio ('45) of the Harvard Fatigue Laboratory have carried on extensive studies of the nutritional status of normal young men under various environmental conditions. Because there are conflicting evidences of deficiency and non-deficiency in the same individual when several biochemical tests are applied, they usually have attached significance to one test only when corroborated by the result of a second. For thiamine a fasting urinary excretion of less than 0.6 μ g per hour and an excretion after a 5-mg test dose of less than 20 μ g in 4 hours was considered to indicate an unsatisfactory thiamine status. Table 4 gives fasting urine values obtained from 1 hour samples collected on the first mornings of 5-day periods during which the women were studied and the mornings following the same 5-day periods. Thus, fasting samples were obtained which reflect the voluntary food intakes of the women before the experimental period and other samples portray the changes consequent to

ingesting the known diet for 5 days. All of the fasting excretions exceeded the 0.6 μg per hour minimum suggested by Johnson, Sargent, Robinson and Consolazio ('45).

Ten of the thirteen determinations before and after ingesting for 5 days a diet providing an average of 1.3 mg of thiamine per day show decreases in the rate of excretion in the urine, in two there were increases, and one pair of values indicate no change. While all of the values following

TABLE 4

Thiamine in fasting samples of urine at start and after 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	FIRST MORNING	AVERAGE DAILY INTAKE DURING 5 DAYS	FOLLOWING MORNING
	<i>days</i>	$\mu\text{g/hr.}$	<i>mg</i>	$\mu\text{g/hr.}$
J.M.	75-79	7.1	1.38	5.7
	173-177	8.4	1.59	7.7
V.K.	95-99	12.8	1.07	8.6
	144-148	7.3	1.20	11.4
V.L.	68-72	6.9	1.50	4.8
	152-156	5.6	1.61	5.6
V.G.	78-82	11.3	1.36	5.4
	161-165	6.6	1.18	8.0
	239-243	9.7	1.18	6.0
	302-306	7.7	1.38	6.0
B.S.	85-89		1.11	4.8
	204-208	7.9	1.32	5.0
	259-263	8.8	1.26	3.6
V.S.	70-74	62.0	1.40	12.4

the 5-day period indicate that the thiamine intakes supplied all the needs of the lactating women's bodies, it seems likely that the intakes of ten of the women were higher before than during the 5-day period.

The excretion values for V.S. are of special interest. This subject was taking commercial vitamin supplements daily (6250 I.U. vitamin A, 3.6 mg of thiamine, 2.1 mg of riboflavin, vitamins D and E, niacin, pyridoxine and pantothenic acid) throughout pregnancy and during the interval between the

tenth day postpartum and the 5-day period during the third month of lactation. That she retained in her body some of this additional intake for more than a day is evidenced by the fact that the fasting urine sample on the first mornings of the period showed an excretion of 62.0 μ g of thiamine per hour, a rate five to ten times greater than that of other subjects. Of the 3.99 mg excreted by V.S. during the 5-day period, 1.02 mg was excreted during the first 24 hours.

SUMMARY

The thiamine intake for 5-day periods, secretion in 24-hour collections of milk, and daily excretion in urine were determined for normal multiparas during the first 10 days postpartum and at various intervals in mature milk production. The intakes of the mothers were comparable, qualitatively, but quantity was determined by appetite. With an average intake of 1.1 mg thiamine per day, the average daily secretion in milk increased from 1 μ g on the first day to 95 μ g on the tenth day and the excretion in the urine ranged from 200 μ g on the first day to 145 μ g on the ninth day. During the period of highest secretion of thiamine in milk, 2-3 months postpartum, thiamine in the milk averaged only 8% of the intake.

The average volumes of the mature milk secreted during 5-day periods between 2 and 10 months postpartum, ranged from 268 to 1020 ml. The average daily thiamine content of the milk ranged from 30 to 162 mg. Of the thiamine intake accounted for during eighteen 5-day periods for ten women, averages of 23 and 8% appeared in the urine and milk, respectively.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

IX. THE UTILIZATION OF RIBOFLAVIN DURING LACTATION ¹

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FOUR FIGURES

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Riboflavin is required in increased amounts for tissue regeneration and for accelerated metabolic activity (Copping, '45) and, although little evidence is available, it has been assumed that actively growing children and pregnant and lactating women have higher requirements than those of other adults (Copping, '45). Sure's ('40) experiments with rats have indicated that the riboflavin requirement for the rearing of young are at least five to six times that required for growth. The Food and Nutrition Board of the National Research Council ('45) has recommended riboflavin intakes per day of 2.0 mg for adult men and women, eating diets providing 3000 kg cal. per day, 2.5 mg for pregnant women, and 3.0 mg for lactating women. Keys and co-workers (Keys, Henschel, Mickelsen, Brozek and Crawford, '44; Keys, Henschel, Taylor, Mickelsen and Brozek, '44) have questioned the allowance

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for normal adult men after studies of men receiving restricted riboflavin intakes. After 5 months on a diet providing 0.31 mg of riboflavin per 1000 cal (0.99 mg of riboflavin per day), no physiological handicaps were found in normal young men. Najjar, Johns, Medairy, Fleischmann and Holt ('44) studied the biosynthesis of riboflavin in man and concluded that riboflavin may not be a dietary essential under all conditions, since synthesis of riboflavin by the intestinal bacteria seemed to be an additional source of this vitamin. Recent work with rats, however, indicates that the absorption of riboflavin from the intestine probably is not an important source of this substance (Obermeyer, Wertz and Emerson, '45; Schweigert, McIntire, Henderson and Elvehjem, '45).

EXPERIMENTAL

During an investigation of the utilization of nutriment from food by lactating women, riboflavin was determined in the diets, milk and urine of normal lactating women.

The subjects of the investigation were multiparas with medical records of good or excellent health and successful nursing. Thirteen women participated in the study. Seven were studied during two consecutive 5-day periods immediately following delivery. Five were observed for 1 day 7 to 9 days postpartum. Nine of the thirteen mothers were studied during seventeen 5-day periods at various intervals during the production of mature milk. All of the diets were comparable, qualitatively, but the amounts of all foods except milk were varied so that the appetites of the women were satisfied. Duplicates of each meal during a 5-day period were composited for analysis. Complete 24-hour collections of milk and urine were obtained. Details of the method of manually expressing the milk and of collecting the food and milk are given in preceding publications (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Davies, '45). In conjunction with many periods during which mature milk was collected, fasting samples of urine were obtained the first morning of the 5-day period and the morning following the period. Each

collection represented excretion for 1 hour, 12 to 13 hours following the last meal.

Sample preparation and analysis

Food. About 400 gm of the ground 5-day food composite was homogenized in a Waring Blendor and the volume made to 500 ml. Of this, 20 ml was used for each of duplicate determinations. Each sample was diluted to 75 ml with water and enough 1 *N* sulfuric acid to make the mixture 0.1 *N* with respect to the acid and autoclaved for 10 minutes at 15 pounds pressure. When cool, the solution was adjusted to pH 4.5 with 2.5 *M* sodium acetate then incubated with clarase overnight (about 16 hours) at 37°C. with toluene. Finally, the sample was made to 100 ml, filtered, and 40 ml of the filtrate used for the riboflavin determination as described for milk (Roderuck, Coryell, Williams and Macy, '45).

Milk. An aliquot of the milk from each expression during a 24-hour period was poured immediately into a jar which was kept tightly covered in a refrigerator. The composite for the day preceding was taken to the laboratory each morning and held under refrigeration pending analysis by the method described in a preceding paper (Roderuck, Coryell, Williams and Macy, '45).

Urine. Collections of urine were combined, preserved with acetic acid (the final solution was 2% acetic acid) and kept under toluene until the 24-hour composite was complete. Each day the composite was taken to the laboratory, the volume checked and aliquots removed for analysis by the fluorometric procedure as described for milk (Roderuck, Coryell, Williams and Macy, '45). Filtration was the only pretreatment required with urine since all the riboflavin was in the free state, so measured amounts of filtered urine were added directly to Florisil columns. Usually 10 ml per liter of urine excreted was used for each determination.

RESULTS AND DISCUSSION

The average intake of riboflavin from the diets, analyzed "as eaten," was 3.1 mg per day, slightly more than the allow-

ance recommended by the Food and Nutrition Board of the National Research Council ('45) for a woman weighing 56 kg. Adjusted on the basis of the weights of the women studied the recommended allowance was 3.2 mg per day (Kaucher, Moyer, Williams and Macy, '46). The average intake of 3.1 mg was accomplished chiefly through two items in the diet: (a) there were large quantities of milk included in the daily menus, more than a quart and (b) liver was fed on the fifth day of each study period, thus increasing the average intake per day. The average of the analytical values for 5-day composites, 3.1 mg, was slightly less than the value of 3.3 mg calculated from tables of food composition (Kaucher, Moyer, Williams and Macy, '46). On the basis of the calculated figures, the average diet of the fifth day provided 51% more riboflavin than the mean intake for the other 4 days.

In table 1 are given the daily milk and urine volumes, the average riboflavin intakes, the daily secretions of total and free riboflavin in milk, and the daily excretions in the urine during the first 10 days postpartum. The averages of the values for each of the first 10 days postpartum are given in table 2.

Parallel with increasing milk production during the first 10 days postpartum, the secretion of riboflavin in the breast milk also increased. In general, riboflavin excretion in the urine also was augmented during the puerperium. Apparently, the diets eaten by the mothers provided for all the maternal needs following delivery and permitted increased production of milk containing equal or greater concentration of the vitamin. As recovery from childbirth and physiologic adjustment to lactation proceeded, greater excesses of intakes over requirements were portrayed by larger amounts in the urine. These contentions are supported by the effects of serving liver on the fifth and tenth days, which in the preceding paper (Roderuck, Coryell, Williams and Macy, '45), was noted to have a possible influence upon the mean concentration of riboflavin in milk on the fifth day and a greater effect on the tenth day. From the averages in table 2, the liver eaten on the

TABLE 1

Riboflavin intakes, excretion in urine and secretion in immature milk during first 10 days postpartum.¹

SUBJECT	INTERVAL POSTPARTUM	VOLUME		RIBOFLAVIN			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	ml	ml	mg	mg	mg	mg
L.F.	1	71	2590	3.3	.02	.01	1.93
	2	200	1275	3.3	.08	.05	0.54
	3	733	2420	3.3	.33	.16	0.82
	4	1122	2075	3.3	.47	.22	1.09
	5	1441	1674	3.3	.53	.24	1.77
	6	1501	1718	3.3	.52	.25	1.85
	7	1596	1340	3.3	.62	.35	1.96
	8	1638	1810	3.3	.63	.35	2.34
	9	1676	1374	3.3	.65	.40	1.92
	10	1872	2220	3.3	.82	.52	3.32
V.G.	1	82	2067	2.6	.01		0.32
	2	177	1689	2.6	.03	.01	0.36
	3	849	1775	2.6	.23	.15	0.65
	4	1413	1323	2.6	.49	.32	0.75
	5	1471	1303	2.6	.55	.30	1.79
	6	1782	1148	2.2	.64	.39	1.56
	7	1630	1230	2.2	.63	.47	1.60
	8	1895	1237	2.2	.77	.57	1.70
	9	1828	1104	2.2	.74	.49	1.51
	10	1770	1075	2.2	.78	.47	1.96
V.K.	1	9	3086	2.4			1.07
	2	90	3651	2.4	.02		1.82
	3	484	2596	2.4	.10		2.51
	4	547	2427	2.4	.13		1.55
	5	560	2331	2.4	.16		2.09
	6	663	2249	2.6	.18	.13	2.08
	7	781	3380	2.6	.28	.18	2.11
	8	775	3840	2.6	.24	.17	1.98
	9	794	3115	2.6	.24	.17	1.92
	10	797	3328	2.6	.24	.17	2.44
V.L.	1	30	1757	3.2			0.87
	2	56	2686	3.2			0.33
	3	353	2759	3.2	.08	.05	0.80
	4	794	1823	3.2	.21	.16	0.84
	5	844	1847	3.2	.27	.18	2.22
	6	955	2710	3.6	.30	.21	2.10
	7	1047	2841	3.6	.30	.18	2.00
	8	1098	2066	3.6	.32	.20	1.81
	9	1118	2151	3.6	.31	.20	1.54
	10	1200	1669	3.6	.36	.22	2.78

TABLE 1—(continued)

TABLE I (continued)							
SUBJECT	INTERVAL POSTPARTUM	VOLUME		RIBOFLAVIN			Urine
		Milk	Urine	Intake	Milk		
					Total	Free	
	days	ml	ml	mg	mg	mg	mg
J.M.	1	35	2182	3.2	0.80
	2	385	1386	3.2	.08	.06	0.83
	3	870	1519	3.2	.27	.18	1.05
	4	1011	1201	3.2	.38	.18	1.00
	5	1121	1283	3.2	.48	.30	2.43
	6	1125	1172	3.3	.48	.32	1.90
	7	1287	1161	3.3	.54	.36	1.88
	8	1136	1520	3.3	.51	.42	1.94
	9	1258	3.3	.48	.35	...
	10	1336	1553	3.3	.65	.54	2.47
C.O.	1	16	1864	3.0	0.42
	2	100	939	3.0	.02	..	0.28
	3	335	1511	3.0	.07	.02	0.42
	4	595	3.0	.17	.09	...
	5	725	..	3.0	.23	.11	...
	6	821	..	2.8	.24	.15	..
	7	798	1470	2.8	.26	.17	1.46
	8	950	1127	2.8	.30	.17	1.36
	9	931	1191	2.8	.29	.16	1.34
	10	660	2.8	.24	.14	...
V.S.	1	6	1869	3.4	1.13
	2	92	1874	3.4	.02	..	0.77
	3	420	2887	3.4	.14	.08	1.15
	4	600	2063	3.4	.21	.11	1.32
	5	697	2065	3.4	.25	.15	2.48
	6	756	1502	3.2	.27	.17	2.05
	7	818	2524	3.2	.30	.18	2.57
	8	837	2237	3.2	.30	.19	2.17
	9	932	1984	3.2	.34	.24	2.26
	10	924	2287	3.2	.36	.23	3.46
M.B.	9	1189	1035	3.6	.49	..	1.67
	8	688	677	3.2	.28	..	0.65
E.L.	8	688	677	3.2	.28	..	2.07
M.S.	7	880	1355	3.4	.31	.24	1.86
G.S.	7	1017	1981	2.6	.33	.20	1.27
F.W.	7	953	1032	2.8	.37	.18	...

¹ The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F., 23.5 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L., 5 hours; J.M. 16 hours; and V.S. 7 hours.

fifth day had only a slight effect in increasing the riboflavin contents of the milk, which were consistent with increases in milk volume. Although the average urine volume decreased on the fifth day, the average riboflavin content was more than doubled. From the fifth to ninth days, excretions decreased but ingestion of liver on the tenth day produced an excretion higher than that on the fifth day. Although the liver may have some effect on the riboflavin secretion on the tenth day, it

TABLE 2

Riboflavin in 24-hour collections of immature milk and urine during the first 10 days postpartum.

INTERVAL POSTPARTUM	MILK			URINE		
	Volume	Riboflavin		Volume	Riboflavin	
days	ml	mg/day	mg/100 ml	ml	mg/day	mg/100 ml
1	36	.01	.020	2202	0.93	.042
2	157	.04	.023	1928	0.78	.040
3	578	.18	.029	2210	1.06	.048
4	869	.30	.033	1819	1.09	.061
5	080	.35	.035	1750	2.13	.127
6	1086	.38	.034	1750	1.92	.119
7	1081	.40	.036	1831	1.88	.114
8	1127	.42	.037	1814	1.74	.106
9	1216	.44	.036	1708	1.74	.114
10	1223	.49	.039	2022	2.74	.147

seems that the amounts in human milk during the puerperium are determined principally by the physiologic processes operating during the development of milk flow and conform to the individual's needs for lactation.

Table 3 presents the average daily values for all of the 5-day periods included in the investigation. For all of the women studied, the volumes of milk secreted during the sixth to tenth day postpartum were more than double the amounts produced during the first 5 days and were larger than the amounts obtained from the same women during later lactation. The augmented milk flow during the puerperium was accompanied by corresponding increases in riboflavin. The relationship of volume and riboflavin content, for the same

TABLE 3

Average daily riboflavin intakes, excretion in urine and secretion in milk during thirty-two 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		INTAKE	RIBOFLAVIN				
		Milk	Urine		Milk		Urine		
					Total	Free			
	days	ml	ml	mg	mg	% intake	mg	% intake	mg
M.B.	72-76	718	691	2.7	.26	10	.15	6	0.69
L.F.	1-5	713	2007	3.3	.29	9	.14	4	1.23
	6-10	1657	1692	3.3	.65	20	.38	11	2.23
V.G.	1-5	798	1631	2.6	.26	10	.20 ¹	8	0.78
	6-10	1781	1159	2.2	.72	32	.48	22	1.67
	78-82	848	1281	3.2	.41	13	.32	10	1.44
	161-165	901	993	3.2	.38	12	.26	8	1.17
	239-244	681	810	3.4	.29	9	.18	5	1.21
	302-306	394	1080	3.1	.18	6	.11	4	1.23
V.K.	1-5	338	2818	2.4	.08 ¹	3	1.81
	6-10	762	3182	2.6	.24	9	.16	6	2.11
	95-99	647	2804	3.0	.24	8	.18 ¹	6	1.82
	144-148	325	1447	3.1	.11	4	.07	2	1.22
V.L.	1-5	415	2174	3.213 ¹	4	1.11
	6-10	1084	2287	3.6	.32	9	.20	6	2.04
	68-72	789	2849	3.5	.25	7	.17	5	1.39
	152-156	680	1756	3.5	.22	6	.13	4	1.26
J.M.	1-5	684	1514	3.2	.24 ¹	8	.18 ¹	6	1.23
	6-10	1228	1352 ¹	3.3	.53	16	.40	12	2.05 ¹
	75-79	708	2265	3.4	.33	10	.27 ¹	8	1.53
	173-177	268	2003	3.3	.11	3	.06	2	1.12
D.M.	45-49	401	1741
C.O.	1-5	354	1438 ¹	3.0	.10 ¹	3	.08 ¹	2	0.37 ¹
	6-10	832	1263 ¹	2.8	.26	9	.15	6	1.39 ¹
B.S.	85-89	1020	1347	3.1	.46	15	.32	10	1.33
	204-208	913	1356	3.3	.45	14	.37	11	1.80
	259-263	676	1219	3.2	.33	10	.25	8	1.75
M.S.	58-62	947	939	3.4	.36	11	.25	8	1.37
G.S.	80-84	899	1078	2.9	.41	14	.31	10	0.95 ¹
V.S.	1-5	363	2152	3.4	.12 ¹	4	.12 ¹	3	1.37
	6-10	853	2107	3.2	.32	10	.20	6	2.50
	70-74	304	1852	3.3	.12	4	.08	2	2.05

¹ Less than five 24-hour collections included in average. For total riboflavin in milk, values were not obtained for first day but values calculated from the concentration in milk of second day are included in average (table 1).

women, continued through the periods of mature milk secretion. Both volume and riboflavin content varied widely for different individuals, but in general, larger secretions of riboflavin corresponded with large productions of milk.

The second 5-day study, or the period 6 to 10 days postpartum, is unusual in that the largest percentage of riboflavin is accounted for at that time. In the first 5 days, it is possible to account for less than 50% of the intake, perhaps owing to an extra demand for riboflavin in parturition. However, it is also possible that all of the riboflavin eaten as liver at noon on the fifth day was not excreted before the end of that day. The mechanism for such a lag in elimination has been suggested by Klein and Kohn ('40) who investigated the synthesis of flavin-adenine-dinucleotide from riboflavin by human blood cells. They found that synthesis occurred *in vitro* and *in vivo*. The concentration of dinucleotide in the cells was increased when supplements of riboflavin were fed, so they suggested that the ability of the cells to synthesize and store the dinucleotide might indicate that the cells serve to limit the loss of this vitamin by renal excretion. This may provide a partial explanation of the higher percentage of riboflavin accounted for in the second 5 days postpartum, the only 5-day period begun immediately following a day in which liver was known to be included in the diet.

The urine excretion during the mature milk study periods averaged 43% of the riboflavin intake, or 1.37 mg per day. In published studies of urinary excretion of riboflavin, wide variation is found among normal individuals on normal diets, but an excretion of 1.37 mg per day is in the highest range of the normal. Strong, Feeney, Moore and Parsons ('41) found that the normal excretion on unrestricted diets appeared to be of the order of 500 to 800 μ g per day. This value decreased rapidly to from 50 to 150 μ g on a dietary intake of from 1 to 2 mg of riboflavin. Since values of less than 200 μ g per day are usually considered to indicate an unsatisfactory intake, he concluded such an intake was, at best, no more than marginal and perhaps insufficient. Axelrod, Spies and Elvehjem

TABLE 3

Average daily riboflavin intakes, excretion in urine and secretion in milk during thirty-two 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		INTAKE	RIBOFLAVIN					Urine
		Milk	Urine		Milk		Free			
					Total	% intake	mg	% intake		
	days	ml	ml	mg	mg	% intake	mg	% intake	mg	
M.B.	72-76	718	691	2.7	.26	10	.15	6	0.69	
L.F.	1-5	713	2007	3.3	.29	9	.14	4	1.23	
	6-10	1657	1692	3.3	.65	20	.38	11	2.29	
V.G.	1-5	798	1631	2.6	.26	10	.20 ¹	8	0.73	
	6-10	1781	1159	2.2	.72	32	.48	22	1.67	
	78-82	848	1281	3.2	.41	13	.32	10	1.44	
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	239-244	681	810	3.4	.29	9	.18	5	1.21	
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V.K.	1-5	338	2818	2.4	.08 ¹	3	1.81	
	6-10	762	3182	2.6	.24	9	.16	6	2.11	
	95-99	647	2804	3.0	.24	8	.18 ¹	6	1.82	
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V.L.	1-5	415	2174	3.213 ¹	4	1.11	
	6-10	1084	2287	3.6	.32	9	.20	6	2.04	
	68-72	789	2849	3.5	.25	7	.17	5	1.39	
	152-156	680	1756	3.5	.22	6	.13	4	1.26	
J.M.	1-5	684	1514	3.2	.24 ¹	8	.18 ¹	6	1.23	
	6-10	1228	1352 ¹	3.3	.53	16	.40	12	2.05 ¹	
	75-79	708	2265	3.4	.33	10	.27 ¹	8	1.50	
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D.M.	45-49	401	1741	
C.O.	1-5	354	1438 ¹	3.0	.10 ¹	3	.08 ¹	2	0.37 ¹	
	6-10	832	1263 ¹	2.8	.26	9	.15	6	1.39 ¹	
B.S.	85-89	1020	1347	3.1	.46	15	.32	10	1.33	
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	259-263	676	1219	3.2	.33	10	.25	8	1.75	
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G.S.	80-84	899	1078	2.9	.41	14	.31	10	0.95 ¹	
V.S.	1-5	363	2152	3.4	.12 ¹	4	.12 ¹	3	1.37	
	6-10	853	2107	3.2	.32	10	.20	6	2.50	
	70-74	304	1852	3.3	.12	4	.08	2	2.05	

¹ Less than five 24-hour collections included in average. For total riboflavin in milk, .. were not obtained for first day but values calculated from the concentration in milk of .. day are included in average (table 1).

per day, averaging 3.1 mg, approximately the amount recommended by the Food and Nutrition Board of the National Research Council. The ratios of thiamine to riboflavin in the diets of the women ranged from 0.3 to 0.5, averaging 0.4, or 1 to 2.5. In figures 1 and 2 the thiamine and riboflavin contents of the 24-hour collections of both immature (fig. 1) and mature (fig. 2) human milk have been plotted against each other. The points for days on which the mothers ate liver are distinguished from other values. While the scatter diagram for the milk of the first 10 days shows a relationship between the amounts of thiamine and riboflavin secreted, the dispersion of the points during the period of rapidly increasing secretion portrays the variation among the individual subjects. For mature milk the diagram shows an almost perfect correlation between the two vitamins when the unusually high riboflavin values resulting from eating liver are disregarded. At all levels of mature milk secretion the average ratio between thiamine and riboflavin is approximately 0.4 (1 to 2.5), the same ratio as the average in the intake.

The ratios of thiamine to riboflavin plotted according to interval postpartum also are shown in figures 3 and 4. During puerperium the ratios for the first 5 days postpartum are low, showing daily secretions of thiamine in the milk to be less than one-tenth of the amounts of riboflavin in the same samples. During the fifth to tenth days postpartum the ratios increased, reaching the proportion of approximately 1 to 4 on the tenth day. Between the tenth and fiftieth days the ratios of thiamine to riboflavin reached the mature level. The ratios for mature milk, show the distribution of the average ratio of 1:2.5 for thiamine to riboflavin. The scatter is a consequence of variations in the composition of the milk of the different women, rather than day-to-day variations in milk of the same women, for in general, the values for the 5 consecutive days in each period for the same women tend to be grouped together.

Fasting sample values, calculated as excretion per hour, are of some significance. Johnson, Sargent, Robinson and

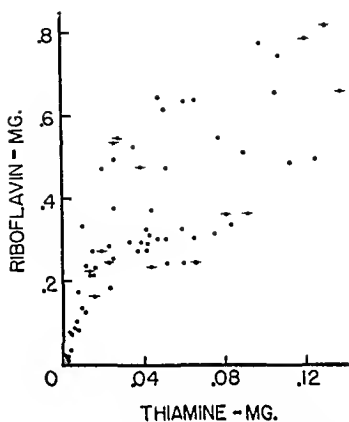


Fig. 1 Secretion of riboflavin and thiamine in immature human milk.

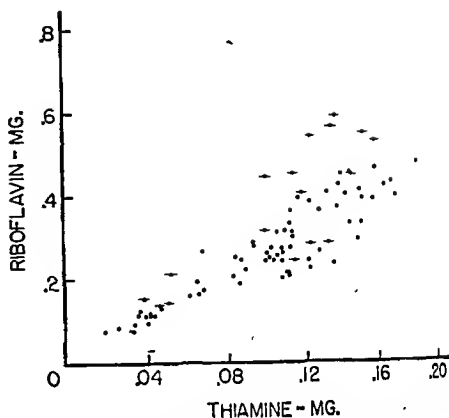


Fig. 2 Secretion of riboflavin and thiamine in mature human milk.

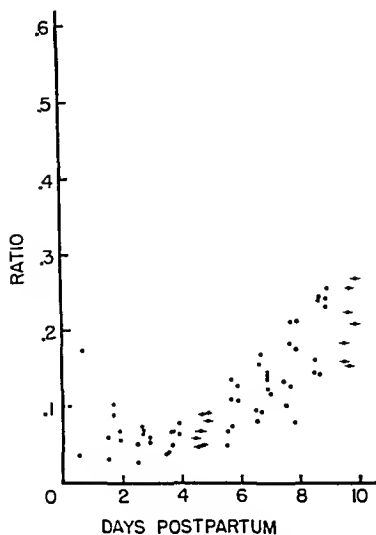


Fig. 3 Thiamine to riboflavin ratio in immature human milk.

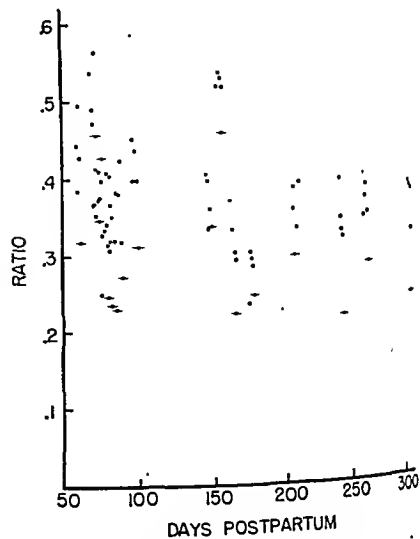


Fig. 4 Thiamine to riboflavin ratio in mature human milk.

Consolazio ('45), consider that a fasting urinary excretion of less than 20 μg of riboflavin per hour and an excretion after a test dose (5 mg of riboflavin) of less than 200 μg in 4 hours is significant and indicates a deficiency state.

Fasting samples were obtained on the first mornings of thirteen 5-day periods of study with lactating women and on the mornings following the experimental period (table 4). Of the fasting samples before the 5-day period, four were less

TABLE 4

Riboflavin in fasting samples of urine before and after 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	FIRST MORNING	AVERAGE DAILY INTAKE DURING 5 DAYS	FOLLOWING MORNING
	<i>days</i>	<i>$\mu\text{g/hr.}$</i>	<i>mg</i>	<i>$\mu\text{g/hr.}$</i>
J.M.	75-79	54	3.4	66
	173-177	52	3.3	72
V.K.	95-99	47	3.0	68
	144-148	17	3.1	70
V.L.	68-72	19	3.5	47
	152-156	26	3.5	41
V.G.	78-82	38	3.2	36
	161-165	15	3.2	42
	239-243	20	3.4	30
	302-306	14	3.1	34
D.S.	85-89		3.1	59
	204-208	32	3.3	49
	259-263	22	3.2	25
V.S.	70-74	154	3.3	49

than 20 μg per hour. The second fasting samples were always taken less than 24 hours after liver had been served, and demonstrate the prolonged effect of the greatly increased intake on the day preceding. In control determinations with normal adults, fasting samples collected 12 hours after a meal containing liver were considerably higher than samples collected 12 hours after a meal containing smaller amounts of riboflavin. For V.S. the fasting excretion preceding the 5-day period was exceptionally high owing to the fact that she had

been taking a riboflavin supplement daily preceding the determination. Since other criteria indicate satisfactory nutritional status with respect to riboflavin for the mothers, it seems likely that fasting excretions of less than 20 μg per hour may be merely effects of low intakes during the preceding 24 hours, rather than evidence of deficiency. It may be that a fasting level of 20 μg per hour is a conservative estimate (may be somewhat too high).

Oldham, Lounds and Porter ('46) have presented evidence that urinary riboflavin excretions vary inversely with the nitrogen intakes, however, no relationship could be found for the mothers who collaborated in this study. Actually all of the subjects were in negative nitrogen balance during the period of 6 to 10 days postpartum, the period of greatest riboflavin excretion. Earlier work (Hunscher, Donelson, Nims, Kenyon and Macy, '33), from this laboratory showed that there is a tendency for lactating women to be in negative nitrogen balance for several weeks after parturition.

In studies of the urinary excretion of riboflavin, wide variation has been found among healthy individuals eating good diets. It is true in general, that only a fraction of the intake is excreted, but increased intake does lead to increased excretion and vice versa. For this reason the 24-hour sample has been concluded by some workers to be of little significance except as indication of the immediate intake, so fasting urine determinations have been investigated by several workers. Feder, Lewis and Alden ('44) believed that the concentration of riboflavin per milliliter of urine was a more constant figure than any other and on the basis of 800 μg per day being the average excretion, they calculated that the range per milliliter should be 0.53 to 0.8 μg . To eliminate borderline cases, less than 0.3 $\mu\text{g}/\text{ml}$ was considered indicative of riboflavin deficiency. The concentrations per milliliter in the present study all exceeded this amount, frequently by very large margins.

The relationship of riboflavin to other dietary factors and to certain physical factors are frequently considered pertinent to the intake of this vitamin. In this study the average value

of 1.1 mg of riboflavin per 1000 cal. exceeds the 0.5 mg per 1000 cal. set by Williams, Mason, Cusick and Wilder ('43) as the minimum daily requirement and also exceeds the value 0.8 mg per 1000 cal. which they found was not associated with any tissue depletion. Too, the subjects' intakes averaged 0.053 mg of riboflavin per kg of body weight, which is substantially higher than the 0.025 mg per kg suggested as a minimum intake by Copping ('45) and also exceeds the 0.035 mg per kg considered minimal by Sebrell, Butler, Wooley and Isbell ('41). The estimated riboflavin intake per square meter of surface area (Boothby and Sandiford, '29) was 1.94 mg.

SUMMARY

Riboflavin was determined in composites exactly representing the food intakes of healthy nursing mothers during 5-day periods and in 24-hour collections of their milk and urine during the same intervals postpartum. The subjects were multiparas and received diets of comparable quality in amounts which satisfied their appetites.

The average daily intake of riboflavin was 3.1 mg per day. During the first 10 days postpartum, riboflavin secretion in milk increased with milk production, from an average of 0.01 to 0.49 mg per day. For the same women, the relationship of volume and riboflavin content continued during the periods of mature milk production. The average daily riboflavin secretion in mature milk was 0.29 mg.

While riboflavin secretion paralleled milk volume, the inclusion of liver in the diet of the fifth day of each 5-day period provided an intake of riboflavin 50% greater than the mean intakes for the other 4 days which produced only slight increases in secretion and excretion on the fifth day postpartum but augmented the amounts of riboflavin in milk and urine collected on the fifth day of all other 5-day periods.

While 3 to 32% of the average riboflavin intakes were found in the immature milk (first 10 days) and 3 to 15% of the intakes was secreted in mature milk, the excretion in urine during respective periods ranged from 12 to 82 and 26 to 61%

of the intake. Together the milk and urine accounted for 15 to 108% of the intake.

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SELF SELECTION OF DIET

III. APPETITES FOR B VITAMINS¹

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In 1933, Harris, Clay, Hargreaves and Ward showed that thiamine-deficient rats developed an appetite for foods containing thiamine, while normal animals showed no such appetite. They were able to produce evidence which clearly indicated that the thiamine appetite was developed because the animals learned that the thiamine-containing diet gave them a beneficial effect or "made them feel better." Later, Richter, Holt and Barelare ('37) described a craving of thiamine-deficient rats for thiamine solutions or yeast, and a less marked craving of riboflavin-deficient rats for riboflavin. Jukes ('38) could not demonstrate an appetite for riboflavin in riboflavin-deficient chickens. Richter and Hawkes ('41) recorded the voluntary intakes by normal rats of thiamine, riboflavin, nicotinic acid, and pyridoxine solutions. The amounts selected were far higher than the requirement — between 0.1–1.20 mg per day.

The present experiments were designed to determine if appetites for thiamine, riboflavin, pyridoxine, and pantothenate could be demonstrated in rats.

METHODS

In each experiment twenty rats were weaned at 21–25 days of age and divided into two groups of five males and five fe-

¹ Contribution no. 602 from the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

males each. Each animal was placed in a cage with a rack containing two cups. During a 3-week control period, one group had both cups filled with a vitamin-containing diet, while the other group had both cups filled with the standard diet.² Vitamins other than the one under study were supplied as pills.³ The cups were alternated in a predetermined random manner, and the amount of food eaten from each cup was recorded daily. During the 3-week experimental period, one of the cups contained the standard diet and the other the vitamin-containing diet. The cups were interchanged in the same random manner as before and the amount of food eaten from each recorded daily. The method of presentation and statistical treatment of the data here reported was previously discussed (Scott and Quint, '46).

RESULTS

The results of the thiamine, riboflavin, pyridoxine and pantothenic acid experiments are recorded in table 1. The thiamine observations confirm the conclusions of Harris et al. All the thiamine-deficient animals began eating the thiamine-containing diet almost exclusively on the first or second day (average 1.3 days) and continued to do so until the sixth to twenty-first days (average 12.2 days). After this they ate from both diets.

With respect to the riboflavin experiment, only six out of ten riboflavin-deficient rats ate the riboflavin diet exclusively. They began to do so on the first to thirteenth day (average

² The standard diet consisted of 24% purified casein (Labco "Vitamin-Free"), 10% hydrogenated fat (Primex), 4% salts (Jones and Foster, '42), and 62% sucrose. The vitamin-containing diets had in addition: Thiamine diet, 5 μ g/gm thiamine hydrochloride; riboflavin diet, 5 μ g/gm riboflavin; pyridoxine diet, 5 μ g/gm pyridoxine hydrochloride; pantothenate diet, 10 μ g/gm calcium pantothenate.

³ Each pill contained approximately: 60 μ g thiamine hydrochloride, 120 μ g riboflavin, 90 μ g pyridoxine hydrochloride, 150 μ g calcium pantothenate, 10 mg choline chloride, 1 mg α -tocopherol, and 55 I.U. Vitamin A and 11 I.U. Vitamin D, as 0.001 ml Natola; all in a dextrin-powdered sugar base. One pill was offered each animal daily. In the thiamine experiment, no thiamine was included in the pills; in the riboflavin experiment, no riboflavin was included, etc.

TABLE 1

petite for thiamine, riboflavin, pyridoxine or pantothenate acid. All data in terms of mean and standard error of the mean.

	GROUP A		GROUP B		DIFFERENCE (IN PER CENT EATEN) ATTRIBUTABLE TO THE VITAMIN DEFICIENCY (A-B)
	Control period	Experimental period	Control period	Experimental period	
<i>Diet</i>	<i>Standard</i>	<i>Choice</i>	<i>Thiamine</i>	<i>Choice</i>	
gain (gm)	15.6 ± 2.2	77.3 ± 4.5	63.9 ± 4.6	39.3 ± 6.3	
total food					
aten (gm)	94.0 ± 4.6	189.2 ± 7.6	155.2 ± 9.8	199.1 ± 14.5	
verage thiamine					
take (μg/day)	0	36.0	37.0	27.3	
ange in percent					
aten from cup 1 ¹		29.8 ± 5.2		8.8 ± 6.9	21.0 ± 8.5
control minus experimental)					
<i>Diet</i>	<i>Standard</i>	<i>Choice</i>	<i>Riboflavin</i>	<i>Choice</i>	
gain (gm)	21.6 ± 3.0	73.5 ± 8.3	85.9 ± 3.1	59.9 ± 7.1	
total food					
aten (gm)	96.4 ± 3.0	210.4 ± 10.4	174.3 ± 10.8	262.5 ± 13.4	
verage					
boflavin	0	34.0	41.5	34.3	
		26.3 ± 11.2		1.8 ± 4.5	24.5 ± 12.1
control minus experimental)					
<i>Diet</i>	<i>Standard</i>	<i>Choice</i>	<i>Pyridoxine</i>	<i>Choice</i>	
gain (gm)	32.2 ± 2.0	75.7 ± 3.0	77.0 ± 2.5	62.8 ± 4.2	
total food					
aten (gm)	105.7 ± 3.7	222.1 ± 7.6	172.8 ± 5.3	267.7 ± 7.0	
verage					
pyridoxine	0	41.9	41.1	36.2	
take (μg/day)		30.9 ± 7.5		8.2 ± 3.6	22.7 ± 8.1
ange in percent					
aten from cup 1 ¹					
control minus experimental)					
<i>Diet</i>	<i>Standard</i>	<i>Choice</i>	<i>Pantothenate</i>	<i>Choice</i>	
gain (gm)	25.3 ± 2.1	42.0 ± 4.5	68.6 ± 3.3	60.9 ± 6.4	
total food					
aten (gm)	120.6 ± 8.6	156.3 ± 10.7	169.7 ± 8.2	237.6 ± 13.3	
verage					
pantothenate	0	37.6	80.8	59.7	
take (μg/day)		-2.8 ± 7.0		3.0 ± 10.7	-5.8 ± 12.8
ange in percent					
aten from cup 1 ¹					
control minus experimental)					

¹ Cup 1 contained the standard diet during the experimental period; a positive change in percent eaten (control minus experimental) is therefore evidence of appetite for thiamine.

4.2 days) and continued until the fourth to twenty-first day (average 15.0 days). In the experiment with pyridoxine eight of ten deficient animals ate the pyridoxine-containing diet starting on the first to fifth days (average 2.4 days) and continued until the fourth to twenty-first days (average 13.9 days). In all three cases, there can be no doubt that an appetite for the vitamin was shown.

In three experiments on calcium pantothenate no appetite for this substance could be detected. The data yielded by one of these experiments are presented in table 1. The results of other experiments in which the choices contained 0 and 5 $\mu\text{g}/\text{gm}$ and 2 and 10 $\mu\text{g}/\text{gm}$ of pantothenate were similar. An experiment outlined in table 2 gave a more definite indication of an

TABLE 2

Effect of flavor on pantothenate appetite.

All data in terms of mean and standard error of the mean.

Diet	GROUP A		GROUP B		DIFFERENCE (IN PER CENT EATEN) ATTRIBUTED TO PANTO- THENATE DEFICIENCY (A-B)
	Control period	Experimental period	Control period	Experimental period	
	<i>Standard or standard plus anise</i>	<i>Choice</i> ¹	<i>Pantothen- ate or pan- tothenate plus anise</i>	<i>Choice</i> ¹	
Wt. gain (gm)	29.9 \pm 3.4	56.3 \pm 10.4	69.1 \pm 3.8	64.9 \pm 6.2	
Total food eaten (gm)	98.5 \pm 5.2	179.2 \pm 17.8	158.3 \pm 8.1	255.5 \pm 8.9	
Average pantothenate intake ($\mu\text{g}/\text{day}$)	0	60.9	75.4	58.6	
Change in per cent eaten from cup 1 (control minus experimental)		23.6 \pm 16.0		- 4.4 \pm 3.3	23.0 \pm 16.3

¹ Half of the animals in each group were given their choice of unflavored standard diet and unflavored pantothenate diet; the other half chose between flavored standard diet and unflavored pantothenate diet.

appetite. Here one of the choices was labeled with anise flavor (10 p.p.m.) which a previous experiment has shown is itself neither liked nor disliked by rats (Scott and Quint, '46). In the control period, one-fourth of the animals were fed the standard diet, one-fourth the anise-flavored standard diet, one-fourth the pantothenate (10 μ g/gm) diet, and the other fourth a flavored pantothenate diet. The choices during the experimental period are described in the footnote to table 2. During this latter period seven of the ten pantothenate-deficient animals ate almost exclusively the pantothenate diet; the other three selected just as definitely the standard diet. Five out of the ten ate a flavored diet. Of the ten normal animals in Group B, none made a definite choice.

DISCUSSION

An appetite as shown in a self-selection experiment may be classed as one of the following types, depending on its origin: (1) simple preferences, (2) learned appetites, or (3) true hungers. The first type is distinguishable because simple preferences have no relation to nutritional value, and are due to flavor, odor, consistency, or some similar quality of the food. Learned appetites are based upon the ability of the animal to learn by experience that a certain food will give him a feeling of well-being. Hungers are appetites that are based directly on physiologic need and require no learning process. The appetite for calories, previously discussed (Scott, '46), could scarcely be other than a true hunger. Richter ('42) and Young ('41) consider all appetites for dietary essentials as being true hungers.

The present experiments show that appetites for foods containing thiamine, riboflavin, or pyridoxine are developed in rats whose diets have been deficient, respectively, in these vitamins. These appetites are not simple preferences, because they cannot be found in normal animals. They must therefore be either learned appetites or hungers. If a difference in nutritional need is accompanied by a difference in appetite, then the appetite is related to the need, but since

hungers are related directly and learned appetites are related indirectly to need, the evidence here reported is not conclusive in deciding which type of appetite is involved in the preferences for foods containing these vitamins. The fact that the appetites are not developed by all animals and are not developed immediately may be considered, however, as limited evidence that the appetites are learned.

The lack of appetite for pantothenate under the same circumstances may be due either to an insufficient stimulus, i.e., incapacity of the animal to appreciate the beneficial effect of this vitamin, or to inability to distinguish the two diets, or to both factors. The previously described repetitious character of eating behavior when diets are identical indicates that identification of a particular diet is not difficult for a rat, and indeed in the case of thiamine, riboflavin and pyridoxine, the fact that repetitious eating occurs allows the possibility that identification of the beneficial diet may have occurred without sensory perception of the vitamin itself. While it is true that flavoring necessarily makes identification easier and thus could encourage repetitious eating, this fact in itself does not prove that inability to distinguish the two diets was the cause of the failure to demonstrate a pantothenate appetite when no flavor was added, for it would seem equally reasonable that a weaker stimulus would require a more positive identification for choice to be made. The stimulus provided by the beneficial effect of pantothenate cannot be altogether lacking because in such case no preference for foods containing pantothenate would occur even when the choices were clearly distinguishable by flavor.

In any case, the pantothenate appetite as demonstrated must be a learned appetite, since it is associated with a flavor or lack of flavor and not with the vitamin itself. The animals must therefore learn what flavor is to be associated with the vitamin and its beneficial effect, while true hungers should not require a learning process. The experiment on flavor and pantothenate confirms the previous observation that rats have no preference for nor dislike of anise.

SUMMARY

In the cases of thiamine, riboflavin, and pyridoxine, an appetite for foods containing the vitamin is developed in animals previously fed a diet deficient in that vitamin. Normal animals do not show such appetites. Appetites for pantothenate cannot be developed in pantothenate-deficient animals unless the choices are labeled with a flavor, and even in this case some animals make the wrong choice. It is concluded that appetite for foods containing pantothenate is a learned appetite and not a true hunger.

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SELF SELECTION OF DIET

IV. APPETITE FOR PROTEIN ¹

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TWO FIGURES

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In a recent study (Scott, '46) it was found that a considerable percentage of rats failed to eat casein, when the dietary choices offered were this protein, sucrose, hydrogenated fat, and a salt mixture. The present studies were undertaken to obtain further evidence on the nature of this failure to eat an essential nutrient.

METHODS

In each of the first three experiments, ten male and ten female rats were weaned at 21 to 25 days of age and placed in individual cages with four food cups all filled with a mixed diet.² The experiments differed only in that different proteins — lactalbumen, fibrin, or egg albumen³ — were used in the diet. Vitamins were given separately as pills.⁴ During

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² The mixed diet consisted of 62% sucrose, 10% hydrogenated fat (Primex), 4% salts (Jones and Foster, '42), and 24% protein.

³ Wilson's fibrin and Borden's lactalbumen were used. The egg albumen was prepared by dissolving commercial egg white, denaturing it in an autoclave, and extracting the product before drying with distilled water, alcohol, and ether successively.

⁴ Each pill contained approximately: 60 μ g thiamine hydrochloride, 120 μ g riboflavin, 90 μ g pyridoxine hydrochloride; 150 μ g calcium pantothenate; 10 mg choline chloride; 1 mg α -tocopherol; and 55 I.U. Vitamin A and 11 I.U. Vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base. One pill was given each rat daily.

a 3-week control period, the amount eaten from each cup was recorded daily and the cups were then interchanged. In a 3-week experimental period, the choices given the animals were sucrose, fat, salt mixture, and the same protein as in the control period, each in a separate cup. The amount eaten of each was recorded and the cups interchanged daily as before. Since it has been shown that the eating behavior of a rat tends to be like that of its littermates, no more than two animals in any experiment were taken from one litter.

In the fourth experiment, twenty males and twenty females were divided into four groups so that each animal had one littermate of the same sex in each of the other three groups. The members of each group were fed one of the following mixed diets in all of four cups during the control period: (1) 24% casein diet; (2) 24% lactalbumen diet; (3) 24% fibrin diet; (4) 24% egg albumen diet. In the experimental period all animals were given their choice in separate cups of these four mixed diets.

In the final experiment, each of thirty rats was placed in a cage with six cups all filled with a diet which consisted of 62% sucrose, 10% hydrogenated fat, 4% salt mixture, and 6% each of casein, fibrin, lactalbumen, and egg albumen. During the 3-week control period, the eating behavior of the rats was observed and recorded, and the cups interchanged as in previous experiments. In the experimental period seven choices, each in a separate cup, were offered the rats. These included sucrose, hydrogenated fat, casein, fibrin, lactalbumen, egg albumen, and salts. Since the amount of salts eaten has been found to be of a different order of magnitude than the other choices, in this case it was not interchanged with the other choices, but offered separately. The other choices were interchanged in the same random manner as during the control period.

RESULTS

The data on eating behavior obtained in the control period of the first four experiments were comparable to those previously presented. It was found that some difference in

growth rate could be observed when different protein sources were consumed (table 1) and the littermate data of the fourth experiment (table 2) showed that this was in part of nutritional origin. The position eating of the animals in the fifth experiment is of some interest. From the left to the right cups, the animals ate the following average per cent of their total food: 18.6, 15.6, 13.3, 13.2, 16.1, 23.2.

TABLE 1

Growth rate and food consumption during the control period.

EXPT	PROTEIN SOURCE	No. of animals	MALES		No. of animals	FEMALES	
			Growth	Food eaten		Growth	Food eaten
			gm	gm		gm	gm
1	Lactal-bumen	10	85.0 \pm 4.7	169.1 \pm 10.1	10	75.3 \pm 3.2	159.8 \pm 5.3
2	Fibrin	10	70.4 \pm 6.2	145.3 \pm 10.7	10	69.3 \pm 2.2	151.4 \pm 7.1
3	Egg albumen	10	75.1 \pm 4.1	152.7 \pm 6.7	10	65.0 \pm 2.8	145.9 \pm 7.1
5	Mixture	15	94.1 \pm 3.1	198.6 \pm 4.9	15	78.5 \pm 1.8	187.2 \pm 4.6

¹ All data in terms of mean and standard error of the mean.

TABLE 2

*Growth rate and food consumption during the control period.
(Experiment 4)*

PROTEIN SOURCE	NO OF ANIMALS	GROWTH		FOOD EATEN	
		gm		gm	
Casein	10	76.5 \pm 4.7		152.9 \pm 11.5	
Lactalbumen	10	86.3 \pm 4.6		156.2 \pm 9.7	
Fibrin	10	87.5 \pm 3.5		165.0 \pm 8.0	
Egg albumen	10	77.3 \pm 4.4		158.1 \pm 7.7	

The results of the experimental periods of the first three experiments and of the seven choice experiments are presented graphically in figure 1. The choices of proteins of the rats in the seven choice experiments are presented in figure 2. Finally, the results of the experiment where a mixed diet was offered are shown in table 3.

TABLE 3
Appetites for mixed diets.

PROTEIN SOURCE IN CONTROL PERIOD	WT. GAIN <i>gm</i>	TOTAL FOOD <i>gm</i>	APPETITE FOR DIET CONTAINING ¹			
			Casein	Lactalbumen	Fibrin	Egg albumen
Casein	85.0±8.3	272.6±15.7	— 4.2±6.1	13.2±10.4	10.4± 9.4	-19.5±2.8
Lactalbumen	79.7±9.5	266.8±19.6	9.0±7.7	8.2± 9.5	1.7± 8.2	-18.9±3.8
Fibrin	76.5±8.0	275.8±15.2	12.2±5.6	0.6±13.0	3.5±11.2	- 9.2±3.2
Egg albumen	85.0±8.1	266.1±16.0	25.1±8.1	-18.3±12.0	13.3± 8.6	-20.2±8.5
Average	81.6±4.1	270.4± 8.0	10.9±3.7	0.9± 5.4	5.4± 4.7	-16.9±2.6

¹ Change in per cent eaten (experimental minus control) from corresponding cup during control and experimental periods.

¹ Change in per cent eaten (experimental minus control) from corresponding cup during control and experimental periods.

DISCUSSION

If the animals in the first three experiments were divided into those which lost or gained weight during the experimental period (Groups A and B, respectively) only the results of the experiment on egg albumen and the seven-choice experiment differed significantly from the previous casein experiment. This is shown in table 4, where the distribution into groups A and B of the rats in each experiment and the probability of such distribution being due to random sampling is shown.⁵ These probability considerations indicate that egg albumen

TABLE 4
Group distribution of animals on self selection.

PROTEIN CHOICE	NUMBER OF ANIMALS IN		PROBABILITY ²
	Group A ¹	Group B	
Casein	34	53	1.000
Lactalbumen	9	11	.774
Fibrin	7	13	.408
Egg albumen	14	6	.006
All four	2	20	.010

¹ Group A lost weight during the experimental period; group B did not.

² Probability that a group distribution as improbable as that shown could arise solely through random sampling.

was avoided more than the other proteins and that in the seven-choice experiments, a large majority of animals found at least one protein they liked.

The differences in weight change and total calories between the data shown in figure 1 and the previous results on casein can be explained in terms of the difference in number of animals selecting protein, and of the greater growth of the animals during the control period in the later experiments. The only result in figure 1 which has no apparent explanation

⁵ Probability was calculated by the method described in a previous paper (Scott, '46). The formula used in that paper was incorrectly stated and should have read

$$P_1 = \frac{n' \cdot}{(n - x) \cdot n'} (.39)^x (.61)^{n-x}.$$

is the tendency of the rats in the fibrin experiment to eat less fat and more carbohydrate than the average.

The reason for the dislike of egg albumen in these experiments was not apparent, although this protein did differ in not being quite so finely ground as the others and it was more nearly tasteless. The avoidance of fibrin in the seven-choice and its acceptance in the four-choice experiment appears inexplicable.

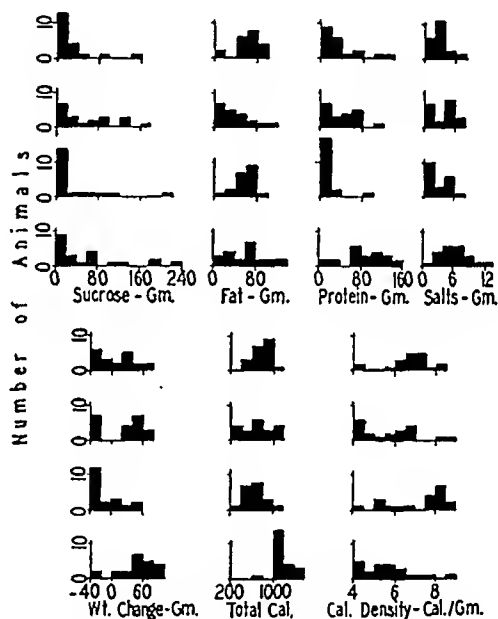


Fig. 1 Weight change and selections of animals when various proteins are offered.

The protein offered was in each case: Top histogram — lactalbumen; second histogram — fibrin; third histogram — egg albumen; lower histogram — casein, lactalbumen, fibrin, and egg albumen simultaneously.

A general principle of self selection may be stated as follows: If N choices are offered a group of animals, the number of possible preferential appetites that can be shown is N minus one. This is true because every dislike leads to an apparent appetite for the other choices, and conversely, each appetite appears to cause a dislike of the other choices. This is well

illustrated in the experiment on mixed diets, where table 3 indicates an apparent appetite for the diet containing casein. But if the dislike of the diet containing egg albumen is real, there should be an apparent average preference for each of the other three diets amounting to $16.9/3 = 5.6$ change in per cent eaten. If 5.6 is subtracted from the ostensible change of 10.9 in per cent eaten of casein, the latter change is no longer significant. If, on the other hand, the preference for the casein diet is considered real, a similar calculation indicates that the dislike of the egg albumen diet is still definitely significant. Thus only a dislike of the diet containing egg albumen was proved in the mixed diet experiment. Furthermore, there appeared to be no general effect of previous diet on appetite.

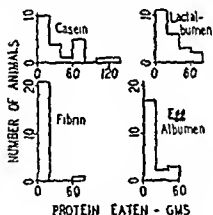


Fig. 2 Selection of proteins when offered simultaneously.

It may be asked whether an appetite for proteins in general exists in the rat, or whether the appetites for casein, lactalbumen, fibrin, and egg albumen are independent of each other. The seven choice experiment seems to indicate the latter is true, and that animals which dislike casein do not necessarily dislike all proteins. The validity of this conclusion was tested as follows: The probability that a rat would not eat casein, lactalbumen, fibrin, or egg albumen when offered individually was 0.39, 0.45, 0.35, and 0.70 as determined experimentally. If these probabilities are independent, the probability that an animal would not eat all four is the product of the individual probabilities which equals 0.043. Accepting the validity of this figure, the probability of occurrence in a random

sample of a distribution such that two out of twenty-two rats would not eat protein is 0.241. Thus the evidence from these experiments must be interpreted as indicating that appetites for different proteins are largely independent.

In a previous discussion (Scott and Quint, '46) it was pointed out that appetites as shown by self selection can be classified into three types depending on their origin. These types were called simple preferences, learned appetites, and hungers. Since there appears to be no appetite for protein in the general sense, and since the nutritional need for protein bears a poor relation to selection of protein, the preferences shown by some animals for various proteins cannot be considered as a true hunger. They may be simple preferences, or they may be learned appetites if one assumes that certain animals are unable to learn to associate beneficial effects with protein. But since it was shown that appetites for different proteins must be essentially independent, while the beneficial effects of the proteins used were approximately equal, a different type of learning, or a learning process dependent on different trivial factors, must be assumed for each individual protein, if a learned appetite is involved. Under these circumstances, the more obvious conclusion that the appetites for various proteins are simple preferences, appears probable.

SUMMARY

When rats were offered their choice of sucrose, hydrogenated fat, salt mixture, and a protein, approximately the same proportion of animals refused to eat the protein if it was casein, lactalbumen, or fibrin. If it was egg albumen, many more refused. If these four proteins were offered simultaneously as choices, only two out of twenty-two animals refused to eat at least one of them. If the choices were offered as part of mixed diets, the animals avoided the diet containing egg albumen, but showed no other marked preferences. It was concluded that the appetites for different proteins were largely independent and that no "appetite for protein" in

the general sense was indicated. The appetite for various proteins, found in some animals, is apparently based on simple preference, although it may possibly be a learned appetite.

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PROTEIN CONCENTRATES AS AMINO ACID
SOURCES FOR THE CHICK:
CORN GLUTEN MEAL, COTTONSEED
MEAL AND PEANUT MEAL

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It has recently become possible to compound a diet of purified or synthetic ingredients in which a particular protein concentrate is the sole source of amino acids for the chick. Such a diet has been used in this laboratory to study the proteins of soybeans (Almquist et al., '42), beef blood (Grau and Almquist, '44a), sesame seeds (Grau and Almquist, '44b) and sunflower seeds (Grau and Almquist, '45b). The present report, which is a continuation of these studies, is concerned with the proteins of corn gluten meal, cottonseed meal and peanut meal.

The ideal way to evaluate proteins as amino acid sources would be to compare the chick's requirement for a particular amino acid with the chemical analysis for that acid in a protein. However, some of the amino acid requirements are still unknown, and the analytical data for the various acids are not all of equal accuracy. Hence feeding trials in which amino acids are used as supplements are essential for the evaluation of protein concentrates.

The literature on the amino acid composition of proteins has been summarized up to 1945 by Block and Bolling ('45). These data, together with some obtained in this laboratory for tryptophane (Kratzer, '44) and methionine (Grau and Alm-

quist, '45a) have served as bases for comparison with the known amino acid requirements (Almquist, '45).

METHODS

White Leghorn chicks which were reared on a commercial-type mash for the first 2 weeks after hatching were weighed every other day during the second week. On the basis of weight and rate of gain, groups of 6 to 12 chicks were selected and given the experimental diets. The birds were housed in electrically heated battery brooders with wire floors; feed and water were supplied ad libitum. The chicks and feed were weighed every other day during an 8-day period. The basal diet consisted of calcium gluconate 8, tricalcium phosphate 2, dipotassium phosphate 0.5, potassium chloride 0.3, sodium chloride 1.0, choline chloride¹ 0.2, inositol 0.1, cholic acid 0.1, crude soybean oil 5, mixed tocopherols² 0.05, and fortified sardine oil, 3000 A-400 D per gm) 0.25 gm per 100 gm diet, and thiamine 1, riboflavin 1, pyridoxine 1, nicotinic acid 3, calcium (d) pantothenate 3, 2-methyl, 1-4, naphthohydroquinone diacetate 1, biotin³ 0.1, folic acid⁴ 1, manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8 and cobalt 0.5 mg per 100 gm diet. Enough of the protein concentrate was added to provide 20 gm of crude protein in the diet, and glucose⁵ was added to a total of 100 gm. The various supplements replaced glucose in the diet. The corn gluten meal contained 45.6% crude protein ($N \times 6.25$), the cottonseed meal 44.4% and the peanut meal 42.7%.

The growth rates were obtained by multiplying the average gain per day by 100 and dividing by the average weight during

¹Choline chloride was provided by Lederle Laboratories, Inc., through the courtesy of Dr. T. H. Jukes.

²Concentrate of natural mixed tocopherols (15%), Distillation Products, Inc.

³Biotin was provided by Merek and Co., through the courtesy of Dr. J. C. Keresteszy.

⁴Folic acid was provided by Lederle Laboratories, Inc., through the courtesy of Dr. E. L. R. Stokstad.

⁵Cerelose.

the experiment, to give the per cent gain per day (Grau and Almquist, '43). The results of supplementation on growth were usually very marked, so that there was seldom any doubt of the significance of the results. Since it was found that when the experiments were conducted with different lots of chicks, the greatest difference in the average growth rates of groups fed identical diets was 0.7%, this figure has been used as the limit of significance.

RESULTS

Corn gluten meal

In table 1, the growth results with the various supplements to the corn gluten meal diet are given. The growth rate was raised from 1.9% (diet 1) to 5.6% (diet 2) by the addition of arginine, cystine, glycine, lysine and tryptophane. The effect on growth of each of these amino acids (together with methionine, threonine and valine) was studied with the corn gluten meal ration. These particular amino acids were studied either because the analytical data indicated a possible deficiency, or because the analytical or requirement data were in doubt.

Arginine. According to Bloek and Bolling ('45), corn gluten meal protein contains 3.2% arginine. When the total protein content of the diet is 20%, the arginine present in the diet is 0.64%, which is less than the requirement of 0.9% (Almquist, '45). As was expected from these data, omission of arginine from the supplement decreased growth (from 5.6%, diet 2, to 4.3%, diet 3).

Lysine. According to the analytical data, about 40% of the required amount of lysine is furnished by corn gluten meal protein. The necessity of supplementation with lysine is clearly shown by the comparison of diet 2 with diet 4, where, when lysine was omitted, the growth was no better than that found with the basal diet (1).

Tryptophane. From chemical analyses (Kratzer, '44), a tryptophane deficiency in corn gluten meal protein was ex-

TABLE 1

Effects of various supplements to the corn gluten meal diet on the rate and efficiency of gain. The concentrate provided 20% protein ($N \times 6.25$) in the diet. The numbers in parentheses are cross-references to other diet numbers. These allow direct comparison of the effects of supplementation by the amino acid at the head of the column.

DIET NUMBER	SUPPLEMENTS TO THE BASAL DIET ¹							NO. OF GROUPTS	AVE. % GAIN PER DAY	GAIN/ FEED
	l(+)-Argi- nine	l(+)-Lysine	l(-)-Trypto- phane ²	l(-)-Cystine	Glycine	dl-Methio- nine	dl-Threo- nine			
1	2	1.9	0.18
2	0.3 (3)	0.55 (4)	0.2 (5)	0.3 (6)	0.8 (10)	.. (12)	..	4	5.6	0.42
3	.. (2)	0.55	0.2	0.3	0.8 (17)	1	4.3	0.33
4	0.3	... (2)	0.2	0.3	0.8	1	1.5	0.16
5	0.3	0.55	.. (2)	0.3	0.8	1	1.7	0.16
6	0.3	0.55	0.2	.. (2)	0.8	2	5.7	0.42
7	0.3	0.55	0.2	0.3 (8)	0.8 (13)	0.2 (14, 15)	..	1	5.6	0.52
8	0.3	0.55	0.2	.. (7, 9)	0.8	0.2	..	1	5.5	0.51
9	0.3	0.55	0.2	0.3 (8)	0.8 (13)	0.1 (14, 15)	..	1	5.8	0.45
10	0.3	0.55	0.2	0.3	.. (2)	2	5.5	0.41
11	0.3	0.55	0.2	0.3	.. (16)	..	0.5	1	5.9	0.44
12	0.3	0.55	0.2	0.3	0.8	0.2 (2)	..	1	5.7	0.51
13	0.3	0.55	0.2	0.3	.. (7, 9)	0.2	..	1	5.9	0.45
14	0.3	0.55	0.2	0.3	0.8	.. (7, 9)	.. (16)	1	5.9	0.51
15	0.3	0.55	0.2	0.3	0.8	.. (7, 9)	.. (16)	1	6.2	0.47
16	0.3	0.55	0.2	0.3	0.8 (11)	..	0.5 (14, 15)	1	5.8	0.44
17	0.3	0.55	0.2	0.3	0.8	..	0.5 (2)	1	5.8	0.44

¹ Expressed as per cent of the diet.

² In some experiments, 0.4% dl-tryptophane replaced 0.2% l(-)-tryptophane.

peeted, and this was confirmed by growth studies. When tryptophane was omitted from the supplements of diet 2, growth was decreased from 5.6% to 1.7% (diet 5).

Cystine. The large variation in the reported cystine content of corn proteins made it desirable to test the effect of this amino acid in the absence, and presence, of methionine. Omission of cystine had no significant effect (compare diet 2 with diet 6, and diets 7 and 9 with diet 8).

Glycine. Like that of cystine, the glycine content of corn gluten meal is not accurately known, and therefore trials were made using glycine supplements. Comparisons of diets 2 and 10, 11 and 16, 7 and 13, and 9 and 13 show no significant effect of the glycine supplement.

Methionine. Corn gluten meal protein was found to contain 2.3% methionine (Grau and Almquist, '45a) when analyzed by the colorimetric method of McCarthy and Sullivan ('42). Since this is probably barely enough to satisfy the methionine requirement (Grau and Almquist, '43), its effect as a supplement was tried. Comparisons of diet 2 with diet 12, diets 7 and 9 with diet 12, and diets 7 and 9 with diets 14 and 15 show that the corn gluten meal diets are not deficient in methionine.

Threonine. The threonine requirement has been in such doubt that some trials were made to determine its possible effect on growth with the corn gluten meal diet. Neither the addition of threonine to diet 2 (as in diet 17) nor to diet 15 (as in 16) gave any increase in growth.

Valine. No effect from valine supplementation was found (diet 2 and diets 14 and 15; diet 12 and diets 7 and 9).

The best rate of growth with the corn gluten meal diets was about 6%, while groups fed supplemented peanut or cottonseed meals showed rates of about 7%, which is the rate obtained with commercial-type rations. It is, of course, possible that some one amino acid is not present in sufficient amount in corn gluten meal; an alternative explanation is that those amino acids which are present occur in proportions which are too different from the optimum to allow best growth.

Cottonseed meal

The amino acid analyses of cottonseed meal point to deficiencies of lysine, methionine, and (possibly) tryptophane. In addition to these amino acids, the effects of supplementing with threonine were studied.

When lysine alone was added to the basal diet, growth was increased from 4.4% (table 2, diet 18) to 5.7% (diets 19 and 20). A similar addition of methionine (diet 21) had no such effect, but the addition of both lysine and methionine (diet 22) allowed a growth of 6.9%.

Threonine supplementation was ineffective (compare diet 24 with diet 23), as was that of tryptophane (diets 25 and 22).

Peanut meal

According to amino acid analyses, the principal deficiencies of peanut meal are those of lysine and methionine, and possibly threonine and tryptophane. Various combinations of these amino acids were added to the basal diet, with the results shown in table 2 (diets 26 through 35).

The basal diet gave poor growth (3.3%, diet 26) which was not increased by the addition of lysine alone (3.1%, diet 27). Methionine, on the other hand, had a definite effect on growth when fed alone (5.8%, diet 28). When the basal diet was supplemented with all four of the amino acids mentioned above, the growth rate was 6.9% (diet 29). When lysine was omitted, growth was decreased only to 5.5% (diet 31) but when methionine was omitted, the resulting growth was 2.7% (diet 32).

The possible supplementary effect of threonine was investigated with diets 29 and 33, 34 and 35, and 34 and 33. Although the difference in growth rate between diets 34 and 33 exceeded 0.7%, the differences within the other two pairs were less than this value, so that the effect of threonine cannot be considered significant.

Omission of tryptophane had no effect, whether threonine was present (diets 29 and 34) or absent (diets 33 and 35).

TABLE 2

*Effects of various supplements to the cottonseed meal and peanut meal diets on the rate and efficiency of gain.
The numbers in parentheses are references to diet numbers.*

PROTEIN CONCENTRATE	DIET NO.	SUPPLEMENTS TO THE BASAL DIET ¹					NO. OF GROUP'S	AVL. % GAIN PER DAY	GAIN/ FEED
		l(+) Lysine	dl-Methionine	dl-Threonine	l(-)Trypto- phane ²				
Cottonseed meal	18	... (19)	.. (21)	1	4.4	0.30	
	19	0.46 (18)	2	5.7	0.38	
	20	0.37 (18)	1	5.7	0.41	
	21	...	0.2 (18)	1	4.3	0.27	
	22	0.46 (18, 19)	0.2 (18) (25)	2	6.9	0.45	
	23	0.37	0.2	.. (24)	..	2	6.3	0.43	
	24	0.37	0.2	0.3 (23)	..	1	6.2	0.43	
25	0.46	0.4	..	0.1 (22)	1	6.6	0.44		
Peanut meal	26	... (27)	.. (31)	1	3.3	0.28	
	27	0.46 (26)	1	3.1	0.26	
	28	...	0.2 (26)	2	5.8	0.38	
	29	0.46 (31)	0.4 (22)	0.5 (33)	0.1 (34)	1	6.9	0.52	
	30	0.46	0.1	0.5	..	1	7.0	0.43	
	31	... (29)	0.4	0.5	0.1	1	5.5	0.40	
	32	0.46	.. (29)	0.5	0.1	1	2.7	0.25	
	33	0.46	0.4	.. (29, 34)	0.1 (35)	3	6.4	0.48	
	34	0.46	0.4	0.5 (33)	.. (29)	2	7.5	0.57	
	35	0.46	0.2 (33)	2	7.0	0.49	

¹ Expressed as per cent of the diet.

² In some experiments, 0.2% dl-tryptophane replaced 0.1% l(-)tryptophane.

SUMMARY

Diets containing corn gluten meal, cottonseed meal or peanut meal were fed to young chicks so that all the 20% crude protein of each diet was provided by one of these concentrates. Additions of various amino acids were made, and the effects on growth and efficiency of gain were noted.

Corn gluten meal required the addition of arginine, lysine and tryptophane in order to increase the rate of growth from 2% to slightly less than 6% per day. Cystine, glycine, methionine, threonine and valine were already present in adequate amounts.

Supplementation of cottonseed meal with both methionine and lysine increased the growth rate from 4% to 7%. The methionine deficiency was found to be less marked than that of lysine, a result which was expected from amino acid analyses.

Peanut meal is lacking primarily in methionine, but is also slightly deficient in lysine.

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THE NUTRITIVE VALUE OF THE PROTEIN OF VARIETIES OF LEGUMES AND THE EFFECT OF METHIONINE SUPPLEMENTATION^{1, 2}

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Although many studies have been made of the nutritive value of the protein of legumes, little attention has been given to the question of whether the nutritive value differs with respect to variety. The proteins of none of the common legumes, peas, lima beans, and snap beans, have been reported to promote growth in the white rat as well as that of soybeans, and it was of interest to find out whether some varieties of the commonly used legumes contain protein of higher nutritive value than others and whether any equal that of the soybean. Furthermore, the observation of Almquist and associates ('42) on the supplementing effect of methionine for soybean protein, those of Woods, Beeson and Bolin ('43) on the supplementary effect of this amino acid for Alaska field peas, and the results of preliminary studies in our own laboratory led to a study of the effect of methionine supplementation and the determination of the methionine content of the legumes studied.

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EXPERIMENTAL

In preliminary studies it had been observed that the use of the common legumes, lima beans, peas or snap beans, as the sole source of protein, at the 8 to 10% protein level, in a purified diet, either failed to promote growth or caused only slight gains. Whenever the diet was supplemented with methionine, however, there was an immediate increase in growth rate; and when the amino acid was removed from the diet there was an immediate loss of weight, indicating that storage of this nutrient had not taken place. None of the other essential amino acids, fed singly, caused a significant response. Woods and associates ('43) have reported marked improvement of growth rate when the protein of Alaska peas was supplemented with methionine. In the present experiment it was further noted that the same conclusion as to growth promoting properties was reached after a 10-day experimental period as after 20 or 28 days. Thus for Kentucky Wonder lima beans the average daily gain during the first 10 days of feeding was 0.2 gm, for the second 10 days 0.4 gm and for the final 8 days of a 28-day period, 0.1 gm. The results of the whole 28-day period, or of any one of the three periods, showed that the nutritive value of the protein of this variety of legume was very poor. Consequently, for the purpose of determining whether the proteins of the varieties of legumes were of good or poor quality, 10-day feeding periods were used. To determine the growth promoting properties of the protein of the varieties of legumes, and the effect of methionine supplementation, on a relative basis, white rats were fed a basal diet for a 10-day period, which was followed by a second 10-day period during which 0.1% methionine was incorporated in the diet, and by a third 10-day period during which 0.6% methionine was supplied. Finally, for a fourth 10-day period, the animals were returned to the basal diet. Three male and three female rats, 22 to 26 days of age and weighing 55 to 65 gm comprised each group.

Legume seeds of known variety were obtained from seed supply houses,³ washed, soaked overnight, cooked in the soaking water as if for human consumption and dried at 50°–60°C. Green snap beans were likewise cooked as for human consumption and prepared for feeding as were the dried seeds. The dried whole beef, reported for the purpose of comparison, was prepared by drying thin slices of raw, frozen, ground lean beef at 55°–60°C. All of the dried products were ball milled to a fine powder before feeding.

Each diet contained salt mixture U.S.P., 4%; sodium chloride, 1%; cane sugar, 5%; and cod liver oil, 1%. Three to 4% corn oil was added, depending upon the fat content of the dried legume, so that the total fat content of the diet was 5 to 6%. The dried ground legume was incorporated in the diet to supply 10% protein, and the total of ingredients was brought to 95% by the addition of starch. When used as a basal, unsupplemented diet, 5% cane sugar was added, and to supplement the diet with methionine, the calculated quantity was incorporated in the 5% of cane sugar. For each 100 gm of diet the following quantities of water soluble vitamins, and other organic substances, were added in an alcoholic solution and dried on the diet: thiamine, 0.5 mg; riboflavin, 1.0 mg; pyridoxine, 0.5 mg; calcium pantothenate, 2.5 mg; nicotinic acid, 2.0 mg; inositol, 0.25 mg; p-aminobenzoic acid, 5.0 mg; choline, 20 mg and biotin 2 µg.

Methionine was determined by the Hess and Sullivan ('43) modification of the McCarthy and Sullivan ('41) method. Hydrolysis of the protein was accomplished by heating with 20% HCl in a sealed tube at 125°C. for 3 hours, as suggested by Hess and Sullivan ('45).

RESULTS AND DISCUSSION

In table 1 average daily gains, average daily food consumption and gains per gram of protein consumed are shown

³ For the supply of seeds, we are indebted to the Associated Seed Growers, Inc., New Haven, Conn., Alexander Forbes and Company, Newark, N. J., and F. H. Woodruff and Sons, Inc., Milford, Conn.

for four varieties of lima beans (*Phaseolus lunatus*), five varieties of snap beans (*Phaseolus vulgaris*) and nine varieties of peas (*Pisum sativum*), when fed with and without methionine. In addition data are shown for one variety of soybeans (*Soja Max.*), for chick peas (*Cicer arietinum*) and, for purposes of comparison, for dried whole beef. The varieties of lima beans, peas and snap beans have been arranged in descending order of the gains per gram of protein consumed when the diet was supplemented with 0.1% methionine.

None of the varieties of lima beans, snap beans or peas showed more than slight growth during the first period on the basal diet and therefore the differences in the nutritive value of the proteins of these varieties were not significant. The largest average daily gains, 0.4 gm for King of the Garden lima beans and for Laxtonian peas, were significantly less than the responses of 1.6 gm and 1.2 gm for soybeans and chick peas, respectively. These marked differences in response were also apparent in the gains per gram of protein consumed.

When 0.1% methionine was added to the basal diets, the response was immediate for all of the varieties of lima beans, snap beans and peas, as well as for the soybeans and chick peas. During the first 24 hours of methionine feeding the gains ranged from 1 gm for some of the varieties of snap beans and peas to 7 gm in the case of the King of the Garden lima beans. Whereas no significant differences in growth response occurred when the basal ration was fed, poor growth being observed for all of the varieties of lima beans, snap beans and peas, supplementation with methionine accentuated the differences in the nutritive value of the proteins so that the gains per gram of protein consumed ranged from 0.9 gm for the Stringless Green Pod snap beans to 3.0 gm for the King of the Garden variety of lima beans. These results lead to the conclusion that there are differences in the nutritive value of the protein of these varieties when they are supplemented with methionine. The gain of 3.0 gm per gram of protein consumed, shown by the King of the Garden lima

VARIETY	PROTEIN CONTENT	BASAL DIET		BASAL DIET + 0.1% METHIONINE				BASAL DIET + 0.1% METHIONINE				BASAL DIET	
		Gain	gm	Food consumption	gm	Gain per gm protein consumed	gm	Food consumption	gm	Gain per gm protein consumed	gm	Change in body wt.	Food consumption
Linia beans (<i>Phaseolus lunatus</i>)	20.00	+ 0.4	6.1	0.7	3.0	9.8	3.0	3.7	12.0	3.1	11.5	- 0.7	11.5
King of the Garden	20.20	+ 0.3	4.8	0.6	1.3	6.5	2.0	2.1	8.2	2.6	6.4	- 1.2	6.4
Fordhook	19.44	+ 0.1	5.3	0.2	1.4	10.6	1.3	2.1	11.1	1.9	8.8	+ 0.2	8.8
Baby Fordhook	19.50	- 0.4	5.4	..	1.3	10.6	1.2	2.2	12.9	1.7	7.4	- 0.5	7.4
Challenger Pole													
Snap beans (<i>Phaseolus vulgaris</i>)													
Sure Crop Wax	25.38	- 0.1	5.5	..	1.6	8.0	2.0	1.6	8.4	1.9	6.4	- 0.7	6.4
Pencil Pod	23.63	+ 0.3	5.6	..	1.5	8.2	1.8	1.6	8.4	1.9	7.7	- 0.2	7.7
Bountiful	19.19	+ 0.1	5.8	0.2	1.3	7.6	1.7	1.8	8.0	2.3	6.6	+ 0.5	6.6
Kentucky Wonder	24.80	- 0.5	4.1	..	0.7	6.9	1.0	2.2	7.6	2.9	6.7	- 0.2	6.7
Stringless Green Pod	22.75	- 0.4	5.2	..	0.5	5.5	0.9	1.9	6.8	2.8	6.7	0.0	6.7
Peas (<i>Pisum sativum</i>)													
Alderman	20.56	+ 0.2	5.3	0.4	2.0	7.7	2.6	1.7	8.5	2.0	7.4	0.0	7.4
Laxton's Progress	29.90	+ 0.3	6.6	0.5	2.3	9.8	2.3	2.8	12.7	2.2	12.4	- 1.5	12.4
Alaska 2 B	26.13	- 0.5	5.3	..	2.0	8.6	2.3	1.4	8.6	1.6	7.9	- 0.2	7.9
World's Record	29.00	+ 0.2	5.0	0.4	1.7	7.6	2.2	1.8	7.3	2.5	5.4	- 0.7	5.4
Laxtonian	27.12	+ 0.4	6.1	0.7	1.9	8.9	2.1	2.0	11.6	1.7	9.1	- 0.2	9.1
Little Marvel	25.60	+ 0.2	5.0	0.4	1.4	7.2	1.9	1.8	7.4	2.4	8.3	- 0.8	8.3
Thomas Laxton	29.00	+ 0.3	6.8	0.4	1.6	8.9	1.8	2.1	10.9	1.9	10.6	- 0.5	10.6
Mammoth Lucious	26.30	0.0	6.3	..	1.6	9.1	1.8	2.0	12.1	1.7	11.5	- 0.7	11.5
Tetou	27.90	- 0.2	5.8	..	0.8	6.8	1.2	1.8	8.8	2.0	7.9	- 0.9	7.9
Soybeans (<i>Soja Max.</i>)													
Bansci	41.50	1.6	7.5	2.1	3.3	11.6	2.8	3.4	13.0	2.6	11.1	+ 1.2	11.1
Chick peas (<i>Cicer arietinum</i>)	21.64	1.2	6.6	1.8	2.5	9.4	2.7	2.8	10.0	2.8	9.8	- 2.4	9.8
Dried whole beef	82.81	3.1	3.7	(No methionine) 11.0	3.0						

* For the 20-day period.

beans, was practically the same as that produced when dried whole beef (table 1) was fed. Therefore, the protein of this variety of lima beans, when supplemented with methionine, and that of dried whole beef, have essentially the same nutritive value. There were no essential differences among the average daily gains of the three other varieties of lima beans, all of them being markedly lower than that of the King of the Garden variety. For each of two varieties, Baby Fordhook and Challenger Pole, the gains per gram of protein consumed were essentially the same, but a somewhat higher efficiency of gain was recorded for the Fordhook variety.

When 0.1% methionine was added to the basal diets containing protein supplied by snap beans, the average daily gains varied from 0.5 gm to 1.6 gm, and the gains per gram of protein consumed, 0.9 gm to 2.0 gm, placed the varieties in the same order as did the daily gains. Both of these ranges of values are essentially the same as those of the varieties of lima beans and peas which showed the poorest responses.

The incorporation of 0.1% methionine in the basal pea diets caused five varieties to show average daily gains, from 1.7 gm to 2.3 gm, and gains per gram of protein consumed, from 2.1 gm to 2.6 gm, which were higher than those of any of the varieties of snap beans and lima beans, except the King of the Garden variety of the latter species. The remaining four varieties of peas showed responses of the same order as those of the snap beans and the poorer varieties of lima beans.

Since only one variety, the King of the Garden, had shown a gain of the same order as that observed for meat, a protein of high quality, and since the animals were still in an actively growing stage, it was desirable to determine whether an increase in methionine percentage would stimulate further growth. Consequently, the level was raised to 0.6%, that proposed by Rose ('37) as meeting the minimum requirements for this amino acid, at the close of the 10-day period during which 0.1% methionine was fed.

Each of the varieties of lima beans showed a marked increase in rate of gain, the average daily gain for three of

them being 2.1 gm to 2.2 gm, as well as in gain per gram of protein consumed. For the King of the Garden variety, the average daily gain became 3.7 gm, the same as that recorded for meat protein in the second 10-day period. The gain per gram of protein consumed remained essentially the same as that observed in the 0.1% methionine period, however, because of the increased food consumption.

Higher average daily gains resulted from the varieties of snap beans, except for the Sure Crop Wax variety, for which the rate of gain remained the same. Two varieties, Kentucky Wonder and Stringless Green Pod, showed increases in rates of gain of 1.5 gm and 1.4 gm, respectively. For these two varieties the gain per gram of protein consumed was almost as great as that calculated for the King of the Garden variety, but the body weights of the animals were much less than those receiving the King of the Garden diet.

In general, the responses when the varieties of peas were fed with the higher level of methionine were inferior to those of lima beans and snap beans. Actually, for two varieties, Alderman and Alaska, there was a lowering of the average daily rate of gain, accompanied by a drop in the gain per gram of protein consumed. An explanation for the lower responses is not apparent. The World's Record and Laxtonian varieties showed virtually no change in rate of gain, but a marked increase in consumption of the Laxtonian diet lowered the gain per gram of protein consumed. For each of the remaining five varieties there was an increase in the rate of gain and for the Teton variety a significant rise in the gain per gram of protein consumed.

When methionine was removed from the diet, and the animals were provided with only the basal ration for a 10-day period, for all of the varieties of lima beans, snap beans and peas immediate cessation of gain and loss of weight occurred, except for one variety of lima beans, the Baby Fordhook, which showed a slight gain. This behavior leads to the conclusion that there is no storage of methionine such as that observed with the vitamins. It is of interest that in many cases

the food consumption remained essentially the same as during the period when 0.6% methionine was fed.

The good nutritive quality of soybean protein is well known, and one variety (Bansei) of this species was included for comparison. During the first basal period (table 1), the average daily gain, 1.6 gm, and the gain per gram of protein consumed, 2.1 gm, were markedly superior to that of any of the varieties of lima beans, snap beans and peas. When 0.1% and 0.6% methionine were fed, the average daily gains exceeded those of all of the varieties except that of the King of the Garden variety. In this instance the responses were of the same order. The higher food consumption by the animals of the soybean group resulted in gains per gram of protein slightly lower than those calculated for the lima bean variety. It is also of interest that the responses when the soybeans were supplemented with methionine are of the same order as those reported in table 1 for dried whole beef. During the basal period the animals on the soybean diet continued to grow in contrast with those on all of the other legume diets, for which losses or, at the most, slight gains, were noted. Thus of the twenty legumes tested, only the soybeans provided sufficient methionine to support growth in the final basal period.

The relatively high nutritive value of chick peas (*Cicer arietinum*), also called "garbanza" (Spanish-speaking countries), "gram" (India) and "ceci" (Italy), observed in earlier studies led to a test of the effect of methionine supplementation of this legume. As noted in table 1, during the first basal period the gain per gram of protein consumed, though not so great as that shown by soybeans, was far higher than that of the best of the responses in the varieties test. During the two periods of methionine feeding the daily gain and the gain per gram of protein consumed were less than the values for soybeans and the King of the Garden variety but better than any of the other varieties except Laxton's Progress peas which showed daily gains of the same order as those of the chick peas. The gains per gram of protein

consumed in the case of the Laxton's Progress variety, however, were somewhat less. During the final basal period the chick peas differed from the soybeans in that there was a loss in weight, probably due to insufficient available methionine for maintenance and growth of animals that were considerably heavier than during the first basal period. The results with chick peas as the sole source of protein in the dietary confirm an earlier observation reported by Mitchell (28).

TABLE 2

Varieties of green snap beans fed at the 10% protein level. Four rats in each group (2 ♂ and 2 ♀). Average daily growth response, average daily food consumption and gain per gm of protein consumed for 10-day periods.

CATEGORY OF INTEREST	SNAP BEANS (PHASEOLUS VULGARIS) VARIETY		
	Tender Green	Giant Stringless	Black Valentine
<i>Basal diet</i>			
Protein content in %	22.19	21.63	20.06
Change in body weight in gm	-0.5	-0.3	-0.3
Food consumption in gm	4.8	4.6	5.5
<i>Basal diet + 0.6% methionine</i>			
Change in body weight in gm	0.0	+ 0.5	+ 0.5
Food consumption in gm	5.5	6.8	7.0
Gain per gm protein consumed in gm	.	0.7	0.7

The protein contents of the cooked dried products, shown in tables 1 and 2, do not bear a relation to the nutritive value of the protein either unsupplemented or when supplemented with methionine. Thus, for example, the protein content of the King of the Garden variety of lima beans is 20.00% and the gain per gram of protein consumed, when 0.1% methionine was fed, was 3.0 gm. In contrast, for the Teton variety of peas, whose protein content is 27.90%, the gain per gram of protein consumed was 1.2 gm.

During the tests of the varieties of legumes in the form of dried seeds, three varieties of green snap beans became

available from the experimental plots of the Department of Vegetable Gardening.⁴ Accordingly, they were tested in the same manner as the varieties of dried legumes. Although the protein content (table 2) of the cooked, dried, green snap beans was within the range of values obtained with varieties in the dried form (table 1), none of the three green varieties promoted growth when fed as the sole source of protein at the 10% protein level. As sufficient quantities were not available for a complete test, only the supplementation with 0.6% methionine was tried. As noted in table 2, body weight was maintained on the Tender Green variety and very slight average daily gains, and gains per gram of protein consumed, resulted with the other two varieties, the gains being definitely less than any of those observed with the dried varieties. Further study will be necessary to ascertain the cause of this difference, which may be due, for example, to the dilution of seed protein by pod protein or to the possibility that a more complete pattern of amino acids develops as the seed matures.

Following the animal experiments just described, the methionine content was determined on all of the legumes studied, except the Fordhook variety of lima beans and the Little Marvel variety of peas. The results are presented in table 3, along with the gains per gram of protein consumed during the first basal period and when the supplement was 0.1% methionine, in order of the decreasing magnitude of the methionine contents of the diets.

The growth response when the basal diets were fed or when they were supplemented with methionine did not bear a direct relationship to the methionine contents of the diets. Thus, the basal diet made from Laxtonian peas contained 0.30% methionine and the gain per gram of protein consumed was 0.7 gm whereas that made from soybeans contained 0.16% and from chick peas 0.14%, yet the gains per gram of protein consumed were significantly higher, being 2.1 gm and 1.8 gm.

⁴ Prof. L. G. Schermerhorn of the Department of Vegetable Crops, Rutgers University, very kindly supplied these varieties.

respectively. These results indicate that the methionine of soybeans and chick peas is more readily available to the rat than that in Laxtonian peas. The failure to obtain more than slight growth on the Laxtonian pea diet is not due to an inadequacy of other essential amino acids because supplementation with 0.1% of crystalline methionine, one-third of the quantity already present in the Laxtonian pea diet, resulted

TABLE 3

Methionine content of legumes and of diets, and growth responses,

LEGUME	NITROGEN CONTENT, OVEN- DRIED BASIS	METHI- ONINE CONTENT, OVEN- DRIED BASIS	METHI- ONINE IN BASAL DIET	GAIN PER GRAM OF PROTEIN CONSUMED	
				Basal diet	Basal diet + 0.1% dl methi- onine
	%	%	%	gm	
Laxtonian peas	4.60	0.83	0.30	0.7	2.1
Stringless Green Pod snap beans	3.89	0.57	0.23	loss	0.9
Bountiful snap beans	3.24	0.45	0.22	0.2	1.7
Baby Fordhook lima beans	3.38	0.41	0.20	0.2	1.3
Kentucky Wonder snap beans	4.24	0.46	0.17	loss	1.0
King of the Garden lima beans	3.32	0.34	0.17	0.7	3.0
Soybeans	7.73	0.75	0.16	2.1	2.8
Challenger Pole lima beans	3.36	0.34	0.16	loss	1.2
Sure Crop Wax snap beans	4.25	0.42	0.16	loss	2.0
Chick peas	3.70	0.32	0.14	1.8	2.7
Pencil Pod snap beans	4.42	0.35	0.13	loss	1.8
Thomas Laxton peas	4.88	0.38	0.12	0.4	1.8
Teton peas	4.74	0.35	0.12	loss	1.2
Laxton's Progress peas	5.05	0.37	0.12	0.5	2.3
Alaska 2 B peas	4.52	0.30	0.11	loss	2.3
Alderman peas	4.53	0.29	0.10	0.4	2.6
Mammoth Luscious peas	4.53	0.29	0.10	loss	1.8

in a gain of 2.1 gm per gram of protein consumed. The same conclusion may be drawn with regard to all of the varieties of lima beans, snap beans, and peas listed in table 3, since even in the case of those which provided the lowest percentage of methionine in the diet, the Alderman and Mammoth Luscious varieties of peas, the quantity was 0.1%, an amount which fed as a crystalline supplement did promote growth.

These observations emphasize the danger that may be encountered in using the results of the amino acid analysis of proteins for the compounding of rations and the desirability of testing the results of chemical and microbiological analyses by animal experiments.

It is of further interest that the methionine provided by the King of the Garden variety of lima beans, along with the supplementary level of 0.1%, made a total of 0.25% in the diet. The gain recorded for this variety was of a high order, 3.0 gm per gram of protein consumed, yet the total quantity of methionine in the diet, some of which was not available, was less than half of the 0.6% level of methionine given by Rose ('37) as the quantity of the crystalline acid which must be fed along with the recommended amounts of the other essential acids and liberal quantities of the non-essential amino acids, to induce optimum growth.

SUMMARY

1. Since none of four varieties of lima beans (*Phaseolus lunatus*), five varieties of snap beans (*Phaseolus vulgaris*) and nine varieties of edible peas (*Pisum sativum*), when fed as the sole source of a 10% protein level, promoted more than slight growth in the white rat, it is concluded that there are no significant differences in the nutritive value of their unsupplemented proteins. The responses were markedly less than those of soybeans (*Soja Max.*) and chick peas (*Cicer arietinum*) fed under the same conditions.

2. The addition of 0.1% methionine to the basal diet caused an immediate growth response and increase in the gain per gram of protein consumed for all of the varieties of lima beans, snap beans and peas, as well as for soybeans and chick peas, and with this supplementation differences in the nutritive value of the protein of these varieties become apparent.

3. When the level of methionine was raised to 0.6%, there was a further growth response and gain per gram of protein consumed except in the case of three varieties of peas for which losses occurred.

4. Of the eighteen varieties of lima beans, snap beans and peas tested, only the protein of the King of the Garden variety of lima beans, when supplemented with methionine, promoted average daily gains and gains per gram of protein consumed of the same order as those obtained with soybeans supplemented with methionine and with dried whole beef.

5. The nutritive value of the protein of chick peas (also called "garbanza," "gram" and "ceci") is almost as high as that of the soybeans.

6. When three varieties of snap beans, picked green, were fed in the cooked and dried form as the sole source of a 10% protein level, they failed to promote growth and the addition of 0.6% methionine resulted in only very slight gains for two of the varieties.

7. The methionine content of the legumes, on the oven-dried basis, ranging from 0.29 to 0.85%, did not have a direct relationship to nutritive value of the proteins.

8. The methionine of soybeans and chick peas is more readily available to the rat than that present in the varieties of lima beans, snap beans and peas studied.

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INFLUENCE OF A SINGLE DOSE OF ALPHA TOCOPHEROL ADMINISTERED TO VITAMIN E DEFICIENT RATS ON THE FIFTEENTH DAY UPON SUBSEQUENT GROWTH ¹

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FIVE FIGURES

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In cooperation with Pappenheimer and his associates (Kaunitz et al., '44; Pappenheimer et al., '44), it was shown that the administration of a single dose of alpha tocopherol to rats on a tocopherol deficient diet delays the onset and decreases the degree of testicular atrophy. The effect of 1 mg alpha tocopherol was most pronounced when given on the fifteenth day of life, less definite on the twenty-eighth day and inapparent when given on the sixth day of life. Similarly, the oxygen consumption of vitamin E deficient rats was influenced over a period of several months when 1 mg alpha tocopherol was fed on the fifteenth day (Kaunitz and Pappenheimer, '43).

The influence of tocopherol on growth has been studied before (Evans, '28; Oleott and Mattill, '36). Rats on a tocopherol low diet show growth deficit several months after birth. It seemed worthwhile to ascertain whether this effect could also be influenced by a single dose of tocopherol given during the lactation period.

¹ Aided by a grant from the John and Mary R. Markle Foundation.

METHODS

The data presented in this study were obtained from a colony of albino rats maintained on two different diets. One group and its ancestry for six generations had been kept on the "Evans-Burr" diet, consisting of: casein, 32 parts; corn-starch, 40 parts; yeast, baker's dried, 10 parts; lard, commercial, 22 parts; salts (Hawk-Oser), 4 parts and cod liver oil, 2 parts.

The second diet (table 1) used was of "semi-synthetic" character and was developed in cooperation with Dr. Charles Slanetz.²

TABLE 1
Composition of tocopherol-low diet used.

BASAL MIXTURE		SUPPLEMENTS OF BASAL MIXTURE	
	%		mg/kg
Lard	10	Thiamine chloride	2
Casein ¹	30	Riboflavin	4
Cerelose	54	Pyridoxine	4
Celluration	2	Nicotinic acid	100
Salt mixture ²	4	Choline	1000
		Vitamin K	4
		Para-amino-benzoic acid	300
		Ca pantothenate	10
		and	
		Percomorphum oil (ml/kg)	0.2

¹ Borden's crude no. 453.

² Hawk-Oser.

The tocopherol content of this diet was repeatedly examined with the Emmerie-Engel reagent (Emmerie and Engel, '38; Devlin and Mattill, '42), according to the internal standard method of Kannitz and Beaver (Kaunitz and Beaver, '44). It contained definitely less than 0.3 mg, and probably less than 0.1 mg tocopherol per 100 gm of diet.

When mothers reared on the "Evans-Burr" diet had a litter, they were immediately placed on the semi-synthetic diet. Eighteen virginal females, reared on this diet, were successfully mated; no "first litter fertility" was observed.

² We are indebted to the Abbott Laboratories for the choline and the synthetic vitamins.

On the fifteenth day of life some of the young were given 1 drop of sesame oil containing the desired amounts of synthetic dl alpha tocopherol,³ the untreated litter mates serving as controls.

Further studies were made on the testes. First the right and, after a further period, the left one was removed under brief ether narcosis. In animals under 60 days of age, no adverse effect on the growth was observed 1 week after semi-castration. In older males, the period of recovery lasted sometimes 2 to 3 weeks. For the studies of the motility of the sperm, epididymis content was examined in saline solution.

RESULTS

I. Effect on growth

Zueker and Zueker ('42) observed that the optimal weight curve of healthy rats maintained on a complete diet is a straight line when the weight is plotted as the abscissa on a logarithmic scale and the ordinate represents the reciprocal value of the age. The slope of the line is constant for rats of the same sex (fig. 1). Deviations from the line of optimal growth represent a weight deficit which can be expressed in per cent of the optimal weight.

Figure 1 demonstrates that the mean weights of male rats on the semi-synthetic diet without additional doses of tocopherol begin to deviate from the optimal growth at the age of roughly 7 weeks; at the age of about 21 weeks, their weight deficit is about 20% and thereafter they actually lose weight. Male rats that had received a single dose of tocopherol of 1, 5 or 27 mg on the fifteenth day show a smaller weight deficit and their weight continues to increase after the twenty-first week.

The individual variations of the results and their statistical evaluation are given in figure 2. The difference between the two groups is statistically highly significant; no differences of

³ Hoffmann-La Roche very kindly supplied us with synthetic dl alpha tocopherol.

Growth of male rats on a
"synthetic" tocopherol low diet

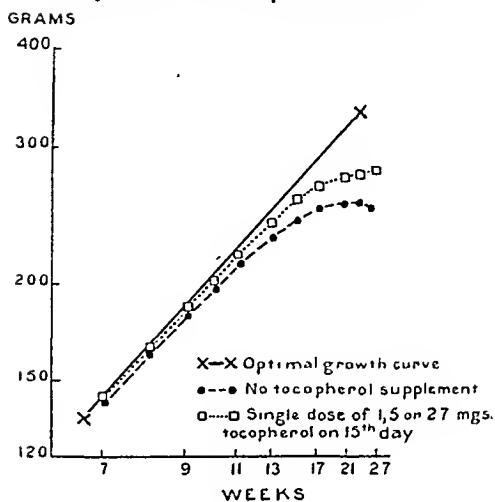


Figure 1

BODY WEIGHT DEFICIT OF MALE RATS ON SYNTHETIC TOCOPHEROL LOW DIET

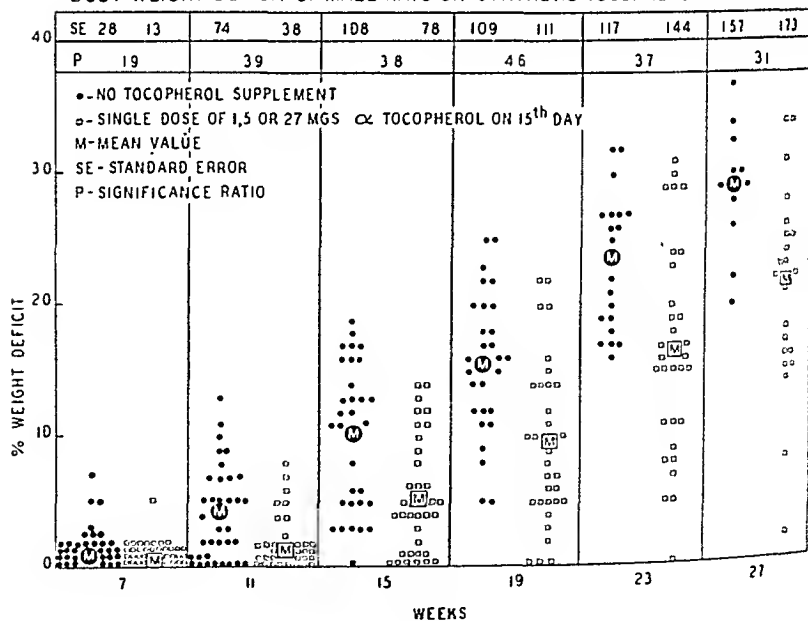


Figure 2

the mean values were noted among those groups that had received graded doses of tocopherol.

In nine male rats of the same colony raised on the semi-synthetic diet augmented by 3 mg synthetic dl alpha tocopherol per 100 gm of diet, the mean weight deficit was similar to that of the group that had received a single dose of alpha tocopherol on the fifteenth day.

Figure 3 demonstrates the weight deficits of male rats raised on the "Evans-Burr" diet.⁴ Again it was noted that the mean weight deficit of the group that received a single dose of 1, 5 or 27 mg tocopherol on the fifteenth day was less than that of the "unprotected" males. The statistical significance of the difference of the mean values is less pronounced than in the groups on semi-synthetic diet (fig. 2), probably because fewer rats on the "Evans-Burr" diet were used.

When synthetic dl alpha tocopherol of from 2.5 to 10 mg per 100 gm was incorporated in the "Evans-Burr" diet, the mean weight deficit observed in a group of nineteen males was 7% after 21 weeks, as compared to 12% in the group with a single dose of tocopherol and with 17% in the "unprotected" males. No differences in the mean values among the males were noted, regardless of whether 1, 5 or 27 mg alpha tocopherol had been given.

While it seems fairer to compare body weight deficits rather than body weight averages, it is to be emphasized that both computations lead to the same conclusions.

II. *Effect on testes*

In figure 4, the weights of one testis of rats on the "Evans-Burr" diet are presented in relation to age and body weight deficit. As previously described (Kaunitz et al., '44; Pappenheimer et al., '44), a single dose of tocopherol given on the fifteenth day delays the onset and decreases the degree of testicular atrophy.

⁴ This colony had been raised from 1941 to 1944 in cooperation with Dr. A. M. Pappenheimer and Mrs. Claudia Schogoleff.

WEIGHT DEFICIT OF MALE RATS ON EVANS-BURR DIET

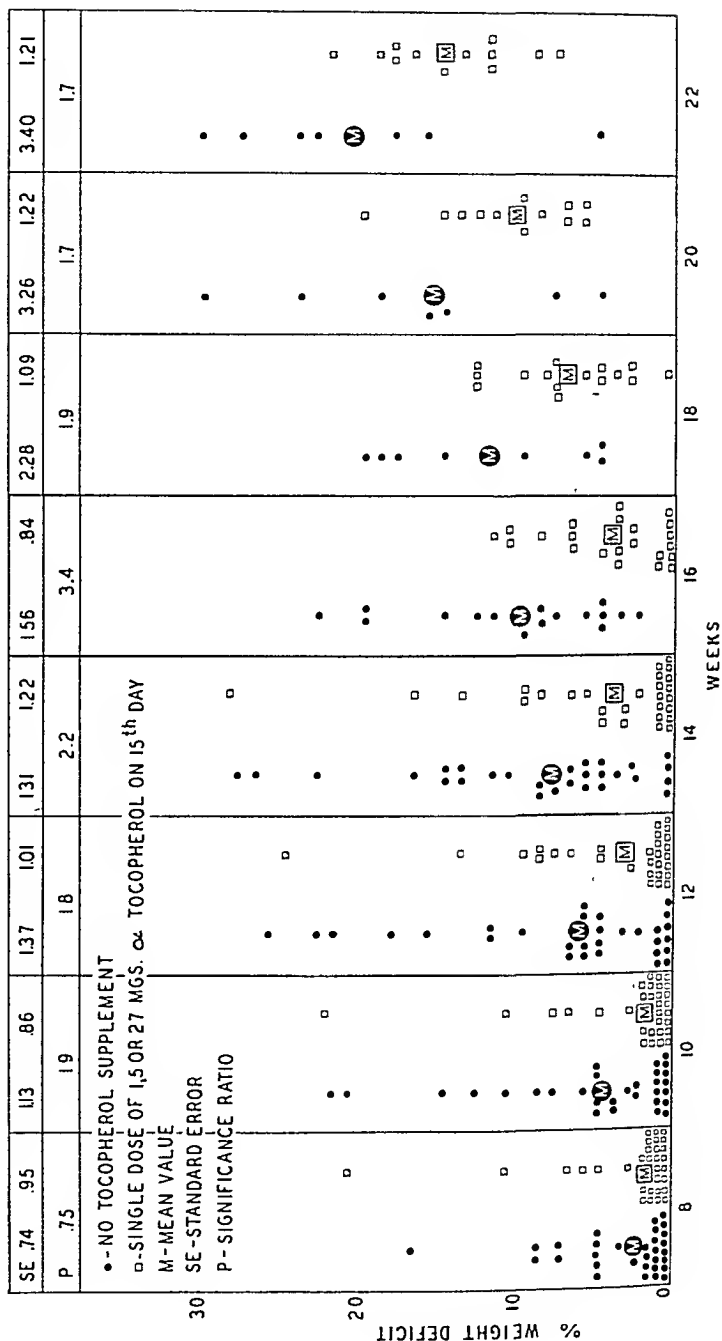


Figure 3

In the age group of 60 to 70 days, only one animal out of five with a testicular weight of less than 700 mg had sustained a moderate body weight deficit. In the age group from 90 to 120 days, fourteen out of twenty-nine animals with testicular weights lower than 800 mg had grown optimally. Body weight

WEIGHT OF ONE RAT TESTIS ACCORDING TO AGE
IN RELATION TO BODY WEIGHT DEFICIT
(DIET I EVANS-BURR)

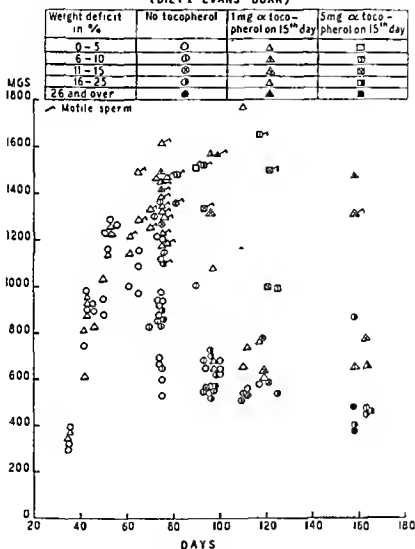


Figure 4

deficit, therefore, did not precede the testicular atrophy in at least 50% of the animals with atrophied testes.

Figure 5 demonstrates the weights of one testis of rats on the semi-synthetic diet in relation to age and body weight. The proportion of animals with atrophied testes and simultaneous body weight deficit is, in the age groups of 70-80 days,

greater than it was among the "Evans-Burr" rats, but there are also in this group animals that had pronounced testicular atrophy despite optimal growth; on the other hand, the number of rats with testes of normal weight and concurrent body weight deficit is considerable. Therefore, this series, too, indicates that body weight deficit does not necessarily precede the testicular atrophy.

WEIGHT OF ONE RAT TESTIS ACCORDING TO AGE
IN RELATION TO BODY WEIGHT DEFICIT
(SEMI-SYNTHETIC TOCOPHEROL LOW DIET)

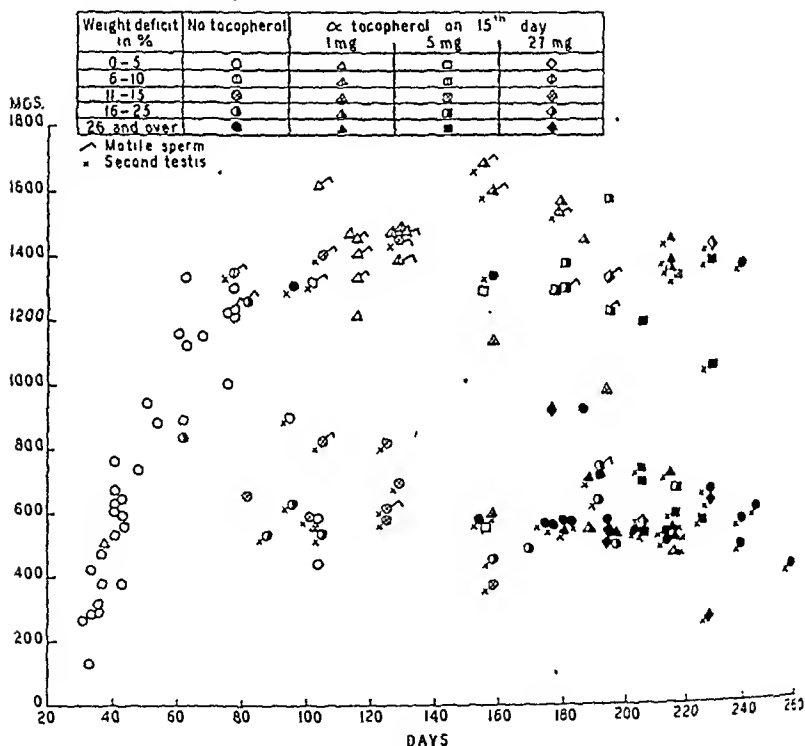


Figure 5

DISCUSSION

Storage and slow utilization of tocopherol cannot fully explain the protracted effect of a single dose of alpha tocopherol, because in the case of the testes (Kaunitz et al., '44;

Pappenheimer et al., '44), it could be proved that the same amount of tocopherol had different effects when given at different ages, and in this study it was observed that doses of from 1 to 27 mg had the same effect on body weight and delay of testicular atrophy when given at the fifteenth day of life. On the "Evans-Burr" diet, however, the degree of testicular degeneration was roughly proportional to the dose given on the fifteenth day. Thus far no explanation can be offered for this latter discrepancy.

If only storage were responsible for the effect of a single dose of tocopherol, one would expect a more pronounced influence from a higher dose and there could not be much difference between the effect of the administration on the sixth and fifteenth days.

The weight deficit caused by the absence of tocopherol does not necessarily antedate the occurrence of testicular atrophy. This permits the conclusion that weight deficit and testicular atrophy are two different, dissociated consequences of tocopherol deficiency in rats.

SUMMARY AND CONCLUSIONS

1. The body weight deficit below the curve of optimal growth was determined in albino rats kept on two different tocopherol low diets.

2. A single dose of alpha tocopherol of 1, 5 or 27 mg administered on the fifteenth day of life to the nursing young significantly reduces the degree of body weight deficit produced by vitamin E deficiency after the seventh week.

3. Determination of the testicular weight in relation to the body weight deficit indicated that testicular atrophy often occurs before body weight deficit is present. Weight deficit and testicular atrophy are therefore dissociated effects of vitamin E deficiency.

4. These observations suggest that in rats there is probably a critical need for tocopherol during the third week of life, since administration of tocopherol during that period exerts a prolonged effect.

ACKNOWLEDGMENT

We wish to acknowledge with gratitude Dr. Alwin M. Pappenheimer's helpful suggestions regarding this work.

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EVIDENCE OF CITRIC ACID SYNTHESIS IN HUMAN SUBJECTS

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ONE FIGURE

(Received for publication April 4, 1946)

It is known from work on experimental animals that citric acid is synthesized in the animal body (Sherman, Mendel and Smith, '36a; Smith and Meyer, '39; Schroeder and Smith, '43; and Class and Smith, '43). No observations of similar synthesis in humans have been reported. An experiment designed primarily to study thiamine (Hathaway and Strom, '46) and riboflavin (Hathaway and Lobb, '46) synthesis and excretion offered the opportunity to investigate citrate excretion on a diet containing no citrates, and on a controlled citrate intake. This paper reports observations which have bearing on the subject of citric acid synthesis in humans.

EXPERIMENTAL

Three women were maintained on a synthetic diet free of citrates for 45 days. After a month's respite they were given, for 21 days, a controlled diet which supplied small amounts of citrates from natural foods. The diets were of similar nutritive value and were considered to be adequate in all respects. Details concerning the subjects and diets have been reported by Hathaway and Strom ('46). Twenty-four-hour urine specimens were preserved with enough glacial acetic acid to maintain an acid concentration of 2 or 3%. Samples were stored under refrigeration until analyzed. The citrate

natural-food diet differed very little from that on the synthetic diet, the daily excretion varying from 506 to 928 mg for subject A; 637 to 1005 mg for subject B; and 481 to 968 mg for subject C. The natural-food diet was shown to contain an average of 797 mg citric acid per day. When this amount of extra citric acid was ingested, urinary excretion of citrates was not affected; nor was it affected when the dietary constituents were supplied as natural foods rather than in the highly purified forms of the synthetic diet. Individual variations in daily citrate excretion of 300 to 500 mg were observed on the rigidly controlled synthetic diet as well as on the natural-food diet. This indicates that in this study citrate excretion was influenced by other factors, independent of dietary constituents.

Relation of citrate excretion to the menstrual cycle

In figure 1 the daily citrate excretion for each subject when on the synthetic diet is plotted graphically to show its relationship to the menstrual cycle. The excretions for subjects A and C show definite cyclic variations, but those of subject B show less marked cyclic variation. In each case the highest excretions occurred about midway in the menstrual cycle and the lowest excretions during menstruation. The midmenstrual peaks exceeded the menstrual levels of excretion by 616 mg, 321 mg, and 583 mg for subjects A, B, and C, respectively. These results confirm those of Shorr, Bernheim and Taussky ('42) who first observed the relationship between citrate excretion and the steroidal reproductive hormones. This hormonal factor may well account for the large individual variations observed in the present study.

Citrate excretion after test doses of ascorbic acid

Purinton and Schuck ('43) observed an inverse relationship between the retention of ascorbic acid by the body and the excretion of citric acid. In connection with another part of the present study (Delaney, '45) test doses of 400 mg of

ascorbic acid were given orally on the forty-second, forty-third, and forty-fourth days of the synthetic diet. The ascorbic acid retention decreased with successive doses, but no variations in citrate excretion were observed beyond those which can be explained by relationship to the menstrual cycle.

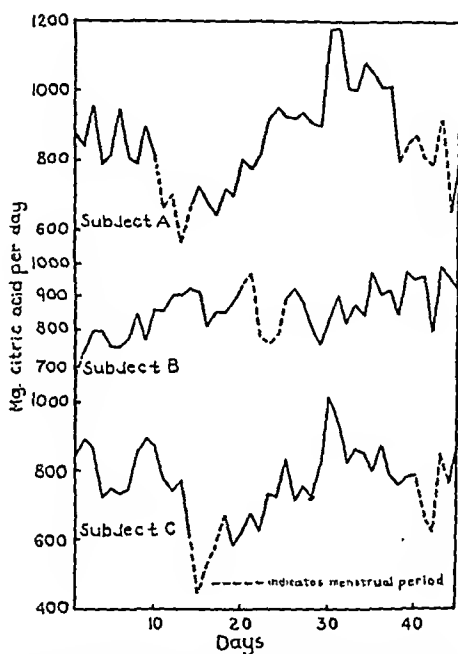


Fig 1 URINARY CITRIC ACID EXCRETION AS RELATED TO THE MENSTRUAL CYCLE

Evidence of citric acid synthesis

The total amounts of citrates as citric acid excreted during the 45-day period on the synthetic diet were 38, 39 and 34 gm for subjects A, B, and C, respectively. This citric acid was necessarily of endogenous origin since there was no dietary source of citrates. Sherman, Mendel and Smith ('36a), Smith and Meyer ('39) and Schroeder and Smith ('43) have reported similar results for balance studies with animals. Endogenous citric acid was considered to be synthesized by the body until Dickens ('41) found that ox and kitten bone con-

tained nearly 500 mg citric acid per 100 gm of fresh bone, and indicated that bone stores had not been ruled out as its source. Class and Smith ('43) and Leonards and Free ('44) demonstrated in animal experiments that the urinary citrates did not originate from reserve stores. A rough calculation can be made of possible bone stores of citric acid in the human. Assuming that the fresh weight of the skeleton of the human adult is 15% of the body weight (Mitchell et al., '45) and that human bone contains citric acid in amounts comparable to that reported by Dickens ('41) for animal bone (approximately 0.5%), subjects A, B, and C would have stores amounting to approximately 43, 45 and 47 gm, respectively. If these stores were the source of the urinary citrates they would have been from 70 to 90% depleted by the end of the dietary period. No decline was found in the level of excretion toward the end of the period which could be attributed to decreasing body stores. Such a great depletion of the citric acid stores in the bones would probably have caused some increase in calcium excretion since the two substances are thought to be closely associated in bone (Dickens, '41; Gomori and Gulyas, '44). No such change in calcium balance was noted.¹ Therefore the results of this study with human subjects support the conclusion drawn from animal experiments that citrates in the urine are largely synthesized by tissues rather than coming from body stores.

SUMMARY AND CONCLUSIONS

Three normal women were maintained for 45 days on a synthetic diet free from citrates and for 21 days on a controlled natural-food diet of similar nutritive value but supplying approximately 797 mg citric acid per day. Urinary excretions of citrates were followed daily.

Individual variations in daily citrate excretion of 300 to 500 mg were found on both the synthetic and the natural-food diets.

¹ A report of the calcium, phosphorus and nitrogen metabolism of the subjects on the synthetic and natural-food diets is in preparation.

EDITORIAL REVIEW

THE ROLE AND EFFICIENCY OF ANIMALS IN UTILIZING FEED TO PRODUCE HUMAN FOOD

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In the face of famine situations throughout the world, measures have been taken in this country to curtail livestock feeding operations in the interests of conserving grains for shipment abroad. It has long been recognized that the production of meat, eggs, and milk from crops which might be consumed direct by man involves a large wastage of the basic food supply as measured in calories. For a complete diet, however, account must also be taken of protein, minerals and vitamins. Animal products are of special value as concentrated and palatable sources of these nutrients. What is their relative nutritional value? How do different animal species compare in efficiency in producing human food? To what extent can foods of plant origin satisfactorily replace milk, meat or eggs in terms of overall nutrition? Though the practical interest in these questions is, doubtless, a temporary one in this country, they will continue to be of basic importance in planning for better nutrition in many countries of the world. Thus, a discussion of them is here presented.

EARLIER STUDIES

In the midst of World War I Armsby ('17), in an article entitled "The Cost of Roast Pig," cited figures on the relative efficiency of the various classes of farm animals in converting

grain into animal products for human food. Later, he ('20) discussed in detail the place of a livestock industry in general, and of various classes of animals in particular, with reference to the most effective utilization of our food resources. His opening sentence indicates a situation paralleling the one we face today. It reads: "The experiences of the great war have forced us to realize as never before that the maintenance of the food supply is the basal problem of civilization." He called attention to the large losses which occur in the conversion of wheat into meat as compared with its direct consumption.

Pearl ('20) classified foods derived from vegetable crops and aquatic animals as primary, and those derived from domestic animals as secondary, making the distinction that the secondary products involve large losses of potential food in their production. His data showed that from 1911 to 1918 the secondary foods furnished 39% of the total calories consumed, indicating that the nation's nutrition was on a wasteful economic basis. Earlier, Armsby, as reviewed by Forbes ('26) had stressed the same point in computing that farm animals consumed 3.9 times as much food on an energy basis as people, and that the grain they ate represented 25 times that required to feed the human population. The latter estimate is borne out by a recent calculation by Harper and Hyer ('43) who further pointed out that Illinois, Iowa and Nebraska alone produce enough corn to meet our calorie needs, yet all but 6% is fed to animals.

The literature contains many reports of feeding trials dealing with the efficiency of farm animals in utilizing feed to produce human food. Data from the earlier reports have been summarized in the monograph by Armsby and Moulton ('25). Later publications which contain pertinent data for the various species are those of Hogan, Weaver, Edinger and Trowbridge ('25); Mitchell et al. ('28); Mitchell and Hamilton ('29); Mitchell, Card and Hamilton ('31); Forbes and Voris ('32); Maw ('33); Edwards ('36); and Hershaw ('38). An especially noteworthy recent publication is the one by Leitch

and Godden ('41), which sets forth data on the relative efficiency of various farm animals as calculated from the intakes in rations recommended by the Ministry of Agriculture of Great Britain for selected levels of production and feeding situations.

The data in these various publications are highly variable for a given species. This is not surprising when one notes the diverse bases on which the calculations are made, and the varying conditions in the feeding trials in question. The feed intake and product are sometimes expressed in pounds and sometimes in gross, digestible, or net calories. In some cases, inedible products are included in the calculations of the energy representing human food produced. Other variable factors which markedly affect the results are: level of production, balance of ration, level of feeding in relation to productive capacity, and length of feeding period under consideration with respect to the normal production cycle for the species in question. Unsound comparisons have been made in some cases because of a failure to appreciate the significance of these different factors. With these variables in mind, the writer has reviewed the various publications reporting feeding trial data, with the objective of getting some useful answers to the questions listed in the first paragraph of this article.

THE EFFICIENCY OF ANIMALS IN ENERGY CONVERSION

The figures shown in table 1 have been arrived at as representing roughly the efficiencies of animals in converting gross energy of their feed into gross energy of edible products. The limitations of gross energy as a measure of feed value are obvious, but no other single measure is applicable to all the species concerned. The digestible energy of a feed as measured with cattle or sheep clearly is not applicable to poultry, or even to hogs. Net energy values are obviously even less suitable. The figures in the table are based on somewhat better than average levels of production, obtained under good feeding practices. The data for the dairy cow are based on

the lactation cycle including a dry period, involving a year's production of 7,000 to 8,000 pounds of milk. Those for the hog cover the period from weaning to slaughter at approximately 230 pounds weight. The data on beef cattle and sheep are limited to feed-lot studies because of the impossibility of measuring the feed intake from pasture or range. The limitations of these data for comparative purposes are recognized. The beef cattle studies in question were made with animals starting around 450 pounds and carried to from 850 to 1,200 pounds in the different experiments considered. The figures for lambs were obtained with animals fed from a

TABLE 1

The recovery in edible products of gross calories of feeds when fed to livestock.

	PERCENTAGE RECOVERY OF GROSS CALORIES IN EDIBLE PRODUCT	
	Estimated range	Estimated single figure as average
Pork	15-25	20.0
Milk (dairy cows)	12-18	15.0
Eggs	6- 9	7.0
Poultry meat	4- 6	5.0
Beef	3- 6	4.0
Lamb	3- 6	4.0

weight of approximately 65 pounds up to a market weight of around 90 pounds, giving no credit for the wool produced. The data for the production of poultry meat cover the period from hatching to market, and those for egg production represent a year's feeding period at a production level of about 150 eggs per year.

The range of values given in table 1 reflects the varying results obtained in different trials, explainable in part by differences in level of production or length of feeding period, or other variables necessarily present even under the narrow range of conditions governing the selection of the basic data. The data in the last column are considered to be the values which might be chosen to represent the data as a whole for a

given species. Of course any set of figures such as those presented in table 1 are subject to challenge because they involve a somewhat arbitrary selection of the data and feeding conditions upon which calculations must be based. It is believed, however, that they present a trustworthy picture of the order of efficiency of the different species as converters of gross feed energy into gross energy in the edible product, under the conditions specified. Clearly, pork production is most efficient, followed rather closely by milk production. The production of eggs rates much lower in efficiency than that of milk, but outranks the production of poultry meat, beef, or lamb.

A particularly important consideration, from the standpoint of the utilization of our potential food resources, is the extent to which the data obtained from feeding trials under the better than average conditions specified may be considered to apply to actual practice. Here, some recently published figures of Jennings ('43) are of special significance. These data, set forth in a condensed form in table 2, take into account the feed consumed and the animal products yielded based on average data for the country as a whole. The reader of this bulletin will appreciate that many assumptions are necessarily involved in the calculations made. The "feed unit" is arbitrarily chosen and the values assigned to certain feeds can be criticized, but so could any other single set of values which are chosen to apply to all feeding operations. Despite the limitations which are inherent in the various calculations involved, it is believed that the data in table 2 present very useful information on the order of efficiency of the production of human food by animals, as now occurring in the country as a whole.

The data for "all feed" indicate, on an entirely different basis, practically the same order of efficiency as shown in table 1. The only marked exception is that the production of poultry meat is given a considerably higher relative rating in Jennings' table. The fact that similar orders of efficiency are revealed from the two widely different bases of calcula-

tion lends confidence to the interpretations which are made as to their significance. The different bases must be kept in mind, however, in any discussion of these data. The data in table 1 indicate what might be expected to result with good feeding practice, while those in table 2 reveal what we are doing at present on a nation-wide basis.

From the standpoint of competition with man's food supply, a comparison based on the entire feed consumption, particularly when expressed as gross energy, is not a fair one because sheep and cattle consume large amounts of roughage, pasture grass, and fibrous by-products inedible by man. Here, they actually increase the human food supply in converting

TABLE 2
Feed units required to produce 2600 Cal. of human food.

ANIMAL PRODUCT	ALL FEED	CONCENTRATES
	lbs.	lbs.
Pork	7.7	7.2
Milk (dairy cows)	9.3	2.3
Eggs	21.9	20.6
Poultry meat	29.9	27.1
Beef	71.6	15.2
Lamb	74.5	4.7

* Adapted from table 36 in a publication by Jennings ('43).

these feeds into meat or milk. In this connection the data headed "concentrates" in table 2 are pertinent. The data indicate that, per unit of feed consumed which might, for the most part, be eaten directly by man, the greatest return can be obtained in milk. Pork production drops below lamb production and the poultry industry becomes the least efficient. Of course, this comparison is faulty, in considering the over-all utilization of potential food resources, to the extent that the pasture grass or hay is frequently produced on land that might be effectively used for the production of crops to be consumed directly.

Further, one must avoid over-emphasis of the significance of these data as a whole. There is much more variation in

the amount of concentrates fed for a given feeding operation in different situations than is the case for total feed. For example, under certain conditions substantial amounts of milk are being produced from roughage alone, while in intensive dairy areas, particularly where pasture is limited, the rate of concentrate feeding is markedly higher than indicated by the average data in table 2. It should also be noted that where a few hogs or chickens are kept on the farm and consume products which otherwise would be wasted such as cull fruits, vegetables and seeds, kitchen wastes, etc., a contribution to the human food supply is made similar to that of cattle and sheep eating roughage. On the other hand, commercial egg production represents the most critical competition for man's food supply of any feeding operation, in that it calls for the feeding of substantial amounts of special vitamin sources such as cod liver oil and riboflavin products needed as supplements to certain human diets.

EFFICIENCY OF ANIMALS IN PRODUCING PROTEIN

Energy conversion is the best single measure of the efficiency of utilization of the feed supply as a whole, but nutritionally there are other considerations which are more important in determining the amounts and kinds of animal products which should be produced. If energy were the sole consideration, there could be no nutritional justification for animal products at all, except milk for the very young, because energy needs can be met from vegetable sources. Animal products have a special value in the diet as rich sources of protein which in general are superior in nutritional quality to vegetable sources. In table 3, data are presented to show the relative efficiency of protein production by various species. These data are taken from feeding trials similar to those considered in setting up table 1. The protein intakes were judged adequate in amount and kind, but not excessive. The first column of figures reveals the order of efficiency of edible protein production in terms of the total ration measured as gross energy. The second column indicates the order of

efficiency in converting feed protein into that for human food. It is noted that on both bases milk production leads, with beef and lamb production showing the lowest efficiency.

TABLE 3

The efficiency of protein production by various animal species.

ANIMAL PRODUCT	EDIBLE PROTEIN PRODUCED PER THERM ¹ OF DIET	EFFICIENCY OF CONVERTING FEED PROTEIN INTO EDIBLE PROTEIN
	<i>gm</i>	<i>%</i>
Milk (dairy cows)	4.0	14.5
Eggs	5.4	9.5
Poultry meat	5.1	13.4
Pork	4.0	14.5
Beef	2.0	8.4
Lamb	1.7	4.4

¹ 1,000 Cal.

ANIMAL PRODUCTS AS SOURCES OF MINERALS AND VITAMINS

Animal products are also of special value in the diet as sources of minerals and vitamins. While the body needs at least thirteen different mineral elements in its food, and probably as many different vitamins, the practical importance of these nutrients is limited to the cases of those which are likely to be lacking in the diet unless attention is given to them in food selection. A list of the minerals and vitamins that are of practical importance, insofar as any of the animal products under consideration is a significant source, is presented in table 5. Since the first essential is to provide a diet with sufficient calories, and since the previous discussion has dealt with the efficiency of calorie production, the mineral and vitamin data are presented as the amounts furnished by a therm (1,000 Cal.) of a given product. This number of Calories represents one-third of the recommended allowance for a moderately active man. Of course, no single animal product is consumed to the extent of one-third of the diet, but the basis selected is useful for comparative purposes.

Since calcium cannot be supplied adequately by any combination of vegetable foods which our people could be expected to eat, its high content in milk makes this food of special importance. A quart of milk a day supplies the full recommended allowance (Food and Nutrition Board, '45) for a growing child except during adolescence, and more than is needed for the adult, except for expectant and nursing mothers. It would take forty-five eggs to supply as much. Other animal products are negligible contributors of calcium. While phosphorus is needed in large amounts by the body, and while animal products are all good sources, this mineral is not considered here because it commonly presents no dietary problem.

The iron needs of the body can be met adequately from vegetable sources alone if the foods are properly selected, but certain animal products represent the most convenient way of obtaining an adequate supply, particularly when the needs are largest. It is noted in the table that eggs far surpass other animal products as a source of this mineral. Beef, poultry meat and lamb supply approximately one-half as much, and pork and milk less than a quarter.

As a source of vitamin A, eggs are markedly superior to its nearest competitor, milk, on a caloric basis. The other animal products are negligible sources. While eggs and milk are important dietary constituents from the standpoint of vitamin A, it is possible to meet body needs from vegetable sources alone.

Nearly half of the thiamine consumed in the current U.S. diet is supplied by animal products. Among these products pork and eggs greatly outrank the others, as is shown in table 4. To meet our thiamine needs without animal products would call for an increased consumption of whole grain or enriched cereals, or a higher level of enrichment.

Riboflavin is very difficult to get adequately from vegetable foods. Milk is the best source, followed by eggs. A quart of milk will furnish 60% of the adult daily allowance. It would require ten eggs to furnish as much. On a calorie basis,

the values for different meat sources range from one-third down to one-eighth of the figure for milk. A decreased supply of riboflavin from animal sources would call for an increased consumption of leafy vegetables, pulses and nuts.

The extent of the need for niacin and its distribution in foods are less well known because the knowledge regarding this vitamin is so recent. Meat products, notably poultry meat, have a special value as a source of this vitamin, in contrast to milk and eggs which are unimportant sources. Whole wheat products are excellent sources, but refined are not, unless enriched. Pulses and nuts also supply liberal amounts.

TABLE 4

Minerals, vitamins and protein supplied by animal products per therm of edible material.

ANIMAL PRODUCT	CA	FE	VIT. A	THIA-MINE	RIBO-FLAVIN	NIA-CIN	VIT. D	PRO-TEIN
	mg	mg	I.U.	mg	mg	mg	I.U.	gm
Pork	15	4	0 ¹	1.6	0.3	7.1	0	26
Milk (dairy cow)	1710	3	2319	0.6	2.5	1.4	38	51
Eggs	342	17	7215	0.8	2.2	0.6	600	81
Poultry meat	83	10	0	0.6	0.9	44.3	0	101
Beef	37	10	0	0.4	0.5	17.5	0	65
Lamb	29	8	0	0.6	0.7	16.6	0	50

¹A zero indicates none or negligible amounts.

Vitamin D is not adequately supplied by any natural food, and vegetable products in general contain none. Where a lack of sunshine makes dietary vitamin D essential, special sources such as cod liver oil or vitamin D milk must be resorted to. Among the animal products, except the special milk mentioned, eggs are the only significant contributor.

The data in table 4 show that on an energy basis, poultry meat supplies more protein than other animal products. Eggs excel beef, milk and lamb; pork ranks low because of the relatively large amount of fat contained in this product. The efficiency of protein production has been considered earlier. It is well recognized that animal protein has a higher nutritional efficiency than most vegetable proteins, but whether

a certain minimum intake of animal protein is essential for optimum nutrition remains an open question.

Any overall nutritional rating of animal products is necessarily subject to the limitation, as indicated in table 4, that no one is superior to another in all nutrients. It seems clear that milk should be given first place, particularly in view of its special value and suitability in the diet of the young. In fact, only this last consideration justifies ranking milk above eggs which actually surpass milk in some of the nutrients under consideration. Pork would appear to deserve a somewhat higher rating than the other meats because of its superiority in the critical vitamins, thiamine and riboflavin.

TABLE 5

The minerals, vitamins, and protein supplied by certain animal products per therm of gross energy consumed.

ANIMAL PRODUCT	ONE THERM OF GROSS ENERGY WILL PRODUCE							
	Ca	Fe	Vit. A	Thia- mine	Ribo- flavin	Niacin	Vit. D	Protein
	mg	mg	I U	mg	mg	mg	I U.	gm
Pork	3.0	0.8	0 ¹	0.30	0.10	1.4	0	5.2
Milk (dairy cows)	256.0	0.9	348	0.09	0.39	0.2	5.7	5.7
Eggs	24.0	1.2	505	0.06	0.15	0.04	42.0	5.7
Poultry meat	4.2	0.5	0	0.03	0.05	2.2	0	5.2
Beef	1.5	0.4	0	0.02	0.02	0.7	0	2.6
Lamb	1.2	0.3	0	0.02	0.03	0.7	0	2.0

¹ As in table 4.

A combined picture of the data in tables 1 and 4 are presented in table 5. Here it is revealed that despite its superiority as a converter of calories in terms of all feed consumed, the hog ranks below the cow and the laying hen as a producer of most of the nutrients which make animal products of special value in the diet. Pork production does, however, excel the other meat-producing operations with respect to most all of the nutrients under consideration.

In considering the competition of animals with man for the basic food supply, it is worth noting that cattle and sheep require no thiamine, riboflavin or niacin in their feed because

these vitamins are synthesized by rumen action. On the other hand, pigs and chickens do require a ration containing these nutrients even as does man. The practical importance of this competition is illustrated by the case of riboflavin. Large amounts and special sources of this vitamin, which is difficult to provide adequately in the diet of man, are required for growth and egg production in poultry. Eggs are nearly as rich in riboflavin as is milk, but the vitamin in the latter can be furnished by the cow herself, whereas that in the egg represents only a small percentage recovery of the riboflavin in the feed.

ORDER OF NUTRITIONAL IMPORTANCE OF LIVESTOCK OPERATIONS

It is clear from the data presented that any proposed adjustment in the production of animal products as a means of conserving the basic food supply should be considered on a differential basis, having in mind both economic and nutrition aspects. On the basis of both efficiency of production of nutrients and of overall nutritive value of the product, milk production should have first priority on the feed supplies available.

An effort should be made to use pasture, hay and silage to the fullest possible extent in feeding dairy cows so as to decrease the need for concentrates which can be used direct by man. At the same time, it must be recognized that the efficiency of milk production increases with the addition of concentrates to a ration of roughage only. Enough concentrates must be allotted to dairy cows to take full advantage of their productive efficiency.

It should be stressed that what has been said about milk production does not apply to butter unless the skim milk is fully utilized as human food—which is the case to a very limited extent. While butter is a highly prized food, it represents, nutritionally, the less important part of milk. All of the protein, minerals, and most of the vitamins, are in the by-product. According to the consensus of present scientific

information, there is no nutritional disadvantage in substituting vegetable fats for butter in the human mixed diet if the vegetable fat is appropriately fortified with vitamin A. Clearly a real effort should be made to recover, for human use, as much as possible of the skimmilk now wasted from butter manufacture. While much of it is used to feed chickens and hogs, this is, nutritionally, a very inefficient use compared to direct consumption.

Nutritionally, egg production ranks close to milk production, but in terms of its competition for man's food supply, it must be rated markedly lower in priority in considering changes which call for a decrease in the production of animal products. The hen should, however, be rated above the hog, the other chief competitor for man's food, as indicated by the data previously presented and discussed. Certainly, considering the poultry enterprise as a whole, the feeds available should be used primarily for egg production rather than for poultry meat. The latter would appear to have the lowest rating of any of the meat-producing operations.

To the extent that beef cattle and sheep can utilize range or other roughage not available to dairy cattle, their production should have a high priority rating, even though they are inefficient producers of nutrients. When large amounts of concentrates are involved in the feeding operations, however, the situation is very different. As far as possible, beef cattle and lambs should be fed grains only to the extent that fattening is necessary to produce meat of acceptable quality. They should be slaughtered at the lighter weights.

The carcass increase that results during the heavy fattening is less valuable for the nutrition of man than that produced earlier because it contains an increased proportion of fat which possesses energy value only. This fact is illustrated clearly by the results of studies with pigs by Hogan, Weaver, Edinger and Trowbridge ('25). These data show that, as the pig grows, more feed energy is needed to produce a pound of protein and that at each additional weight, relatively more fat and less protein tissue is formed. The highly

fat carcass also contains relatively less of the minerals and vitamins which make meat of special value in the diet. There is no nutritional justification for feeding meat animals to produce fat, for vegetable fats will serve as well in the diet. Further, much of this fat of highly fattened animals is wasted insofar as human consumption is concerned. This is particularly true in the case of sheep and cattle.

OTHER FACTORS TO BE CONSIDERED IN EVALUATING THE LIVESTOCK INDUSTRY

The data here presented relate solely to the efficiency of feed utilization. Production per acre and per unit of labor also require consideration in rating the various livestock operations. Data summarized by Hill ('43) indicate that milk should be ranked first on these counts also, considering the production of nutrients of special importance to man.

It is recognized that in the foregoing discussions of animal efficiency the animals have not been given credit for non-food products. Some of the feed wastage is recovered in manure, which indirectly is an important contribution to the food supply through the maintenance of soil fertility. Lambs have been given no credit for the wool they produce. Slaughtered animals contribute hides, inedible by-products for animal feed, and are a source of other important special products. These secondary contributions must be taken into account in fully assessing the place of a livestock industry in the agricultural and industrial economy.

SUMMARY

Both animals and man draw on the same basic food supply — the products of the soil. While the feeding of animals results in a concentration of food nutrients into more palatable and more digestible forms for human use, it also involves a wastage of potential food supplies in the process. Any adjustment of livestock production in the interests of better nutrition for more people should be on a differential basis, recognizing differences among species and feeding operations.

with respect to (a) efficiency of human food production, (b) consumption of foods directly useful to man, and (c) overall nutritive value of the animal product. Taking these factors into account, it is concluded that:

1. Market milk production should have first priority on feed supplies available. Butter production rates the same priority only to the extent that the skimmilk is recovered for human use.

2. Egg production should be ranked above pork production, but broiler production deserves only a very low rating.

3. To the extent that beef cattle and sheep utilize range and other roughage not available to dairy cattle, their production should have a high priority rating, but this is not true where any large consumption of concentrates is involved. The heavy fattening of meat animals is a very inefficient use of the basic food supply in relation to human needs.

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THE ADEQUACY OF INTRAVENOUS PARTIAL ACID HYDROLYSATES OF CASEIN AND FIBRIN FOR NITROGEN BALANCE IN DOGS

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THREE FIGURES

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In intravenous studies with dogs (Risser, Schenck and Frost, '46a, '46b), partial acid hydrolysates of casein fortified with cysteine hydrochloride monohydrate at about 2.8% of the dried hydrolysate gave nitrogen balance at about the same minimum levels of nitrogen as were needed for complete acid hydrolysates of casein fortified with both cysteine and tryptophane, i.e., about 140 mg nitrogen per kilo per day for each. This appeared as presumptive evidence that the peptide fraction of partial acid hydrolysates of casein is well utilized on injection. In further studies with hypoproteinemic dogs (Frost, Heinsen and Olsen, '46), wherein high levels of nitrogen were given intravenously, i.e., 600 mg N per kilo per day, fibrin hydrolysate was utilized far more completely than casein hydrolysate fortified with either cystine or cysteine hydrochloride monohydrate at levels of about 2% of the dried hydrolysate. A comparison of the essential amino acid content of the two hydrolysates was reported in the latter paper.

The object of these experiments was to maintain dogs over a period of many weeks on minimum levels of the partial acid hydrolysates of casein and fibrin. Casein hydrolysate was used "as is" and with an addition of cystine to give a level only slightly below that present in fibrin hydrolysate. Since casein

hydrolysate was shown by analysis to contain more methionine than fibrin hydrolysate, this experiment would show that any superiority of fibrin hydrolysate over casein hydrolysate was not due to a difference in sulfur amino acid content, and must, therefore, be explained on another basis.

The long-term intravenous use of fibrin hydrolysate at a low level of nitrogen input provided data which appeared suitable for application of the graphic method of Allison and Anderson ('45) for estimation of the nitrogen balance index of the preparation.

METHODS

Adult female dogs were placed on a highly purified non-protein diet as follows: 73 gm sucrose, 20 gm lard, 3 gm corn oil, 0.5 gm Haliver Oil, 4 gm U.S.P. salt mixture I, 0.1 gm choline chloride, 1 gm agar, 0.6 mg thiamine hydrochloride, 0.6 mg riboflavin, 12 mg nicotinamide, 0.4 mg pyridoxine hydrochloride, and 1.2 mg calcium pantothenate. The diet supplied 5.1 cal. per gm. To avoid possible vitamin deficiencies during periods of decreased voluntary food intake, biweekly injections of a specially prepared B-complex solution were made to supply per week per kilo body weight: thiamine 0.5 mg, riboflavin 0.5 mg, nicotinamide 15 mg, pyridoxine 0.3 mg, and calcium pantothenate 1 mg. Percomorph liver oil was given by drop during the long injection periods at a rate of 2000 units of vitamin A and 200 units of vitamin D per week. A concentrate of vitamin B₁₂¹ was given orally equivalent to 10 gm of 70% alcohol-insoluble liver extract per week.

Food consumption was recorded daily. An amount of the non-protein diet calculated to supply 80 cal. per kilo per day was offered and any portion not consumed was accounted for. Preliminary to beginning an experiment the dogs were placed 2 to 4 weeks on the above diet supplemented with dried fibrin in an amount equal to 100 mg N per kilo body weight per day, a level previously shown to be adequate for nitrogen balance.

¹ Supplied by Dr. F. C. McIntire.

The standard weight of each dog was taken as the kennel weight at the time the experiment was begun and calculations for nitrogen intake were made on the basis of this standard weight. Since the dogs were fed a highly nutritious and varied diet in the kennel, they were generally at maximum weight at the time they were shifted to the basal diet.

For injection, the dogs were placed on a convenient body support with their legs hanging in natural downward position and held lightly by leather straps. Injections were made into the cephalic and saphenous leg veins in rotation, so that any single vein was used only once in 4 days. Injections were carried out in a regular 2-hour period each morning. The rate of injection ranged from about 0.7 to 1.3 mg nitrogen per kilo body weight per minute depending on the nitrogen input.

Seven-day periods for injection and collection of excreta were observed. The feces were labeled with carmine at the end of each period. The feces and urine for each period were thoroughly mixed in a Waring blender for convenience in sampling. It was determined by preliminary experiment that this procedure gave total nitrogen excretion values comparable to the values obtained by separate analyses of the urine and feces. Nitrogen determinations were made by macro-Kjeldahl, using 10 ml samples.

The hydrolysates used were prepared from commercial casein and fibrin essentially according to the method of White and Elman ('42). These hydrolysates contain about 0.5 and 1% tryptophane, respectively, on a dry basis.⁴ The degree of hydrolysis effected by the conditions of hydrolysis have been studied by Frost and Heinsen ('45). The particular preparations used herein represent 6-hour hydrolysis with 2.8 N sulfuric acid and contain about one-third of the amino acids in free form. The solutions used were sterile-filtered and were non-pyrogenic by U.S.P. test. All solutions contained 5% protein hydrolysate corresponding to about 0.7% nitrogen. Dextrose was added, where indicated, at a 5% level. Cystine was added at 0.08%, the maximum of solubility. This

represents an addition of about 1.6% cystine on the basis of the dry hydrolysate and a ratio of 1 gm of the amino acid to about 9 gm of total nitrogen. Since the casein hydrolysate on a dry basis contained about 0.6% cystine, the total cystine content was about 2.2%.

It is generally recognized that many factors influence the minimum level of protein intake needed to maintain nitrogen equilibrium. These factors have been well described by Melnick and Cowgill ('37). In the present study it was decided to approach a state of nitrogen equilibrium from the side of negative balance, as recommended by the above authors.

EXPERIMENTAL

Casein hydrolysate

Two dogs which had been kept many months on experiment at near maintenance levels of nitrogen were given casein hydrolysate. Dog 7 was started at a level of 105 mg N per kilo and dog 8 at 125 mg N per kilo. Both dogs lost weight rapidly. Weight loss continued even when the nitrogen intake was increased to 130 and 160 mg N per kilo, respectively. Dog 7 developed an infection and died at the end of the fifth week. Dog 8 declined continually and died after 9 weeks' injection.

Dogs 10 and 11 were given casein hydrolysate with added dextrose and cystine. The level of nitrogen was increased from 120 mg to 140 mg and finally to 160 mg N per kilo per day. Both dogs were in severe negative balance throughout and the experiment was discontinued in order to spare the dogs. Biopsy samples were obtained from the livers of dogs 10 and 11 after 5 and 3 weeks, respectively.

A further experiment was conducted with dog 12 in which casein hydrolysate was injected at 160 mg N per kilogram for a period of 4 weeks. A negative balance of 8, 4, 4, and 2 gm N was determined in each of the 4 weeks successively. Body weight fell from 5.35 kg to 5 kg in the same period.

Fibrin hydrolysate

Dogs 3 and 5, which had been maintained for more than a year on near maintenance levels of nitrogen, either oral or injected, were placed on fibrin hydrolysate at levels well below the previously established maintenance level of about 120 mg N per kilo per day. Both dogs were held at levels below 100 mg N per kilo for 5 weeks during which there was gradual loss of weight. The negative balance during this period was consistently small in both dogs. On increasing the input to 110 mg N per kilo, both dogs went into slight positive balance but weight loss continued. Food intake was constant but somewhat subnormal. After 8 weeks' injection, a preparation containing fibrin hydrolysate plus dextrose was introduced to see if this combination would stem the weight loss and improve nitrogen utilization. When no advantage was seen after 2 weeks, the level of nitrogen was increased to 120 mg per kilo per day. However, weight loss continued and after 2 weeks more both dogs were given 100 mg cystine daily by capsule. In the ensuing 2 or 3 weeks, the dogs showed a small weight response and apparent improvement in nitrogen utilization.

Dog 3 became severely ill with a respiratory infection and was sacrificed at the beginning of the fifteenth week of injection after 2 days' illness. The dog was autopsied to determine whether or not liver damage was apparent. A liver biopsy was performed on dog 5 at the end of the fifteenth week of injection to compare with the autopsy samples from dog 3.

RESULTS

Records of nitrogen and weight balance for four dogs which received the various casein hydrolysates are shown in figure 1. The record of dog 12 receiving casein hydrolysates unfortified is not shown, but was comparable to the above in that nitrogen and weight balance were not attained.

Weight and nitrogen balance records for dogs 3 and 5, which received fibrin hydrolysate over 14-15 weeks, are shown

in figure 2. The levels of nitrogen given per kilo body weight, shown in parenthesis, were calculated on the standard kennel weights of the two dogs, which were 7.16 kg for dog 5 and 6.6 kg for dog 3. Nitrogen input calculated on the changing weights of the dogs shows an increasingly higher input per

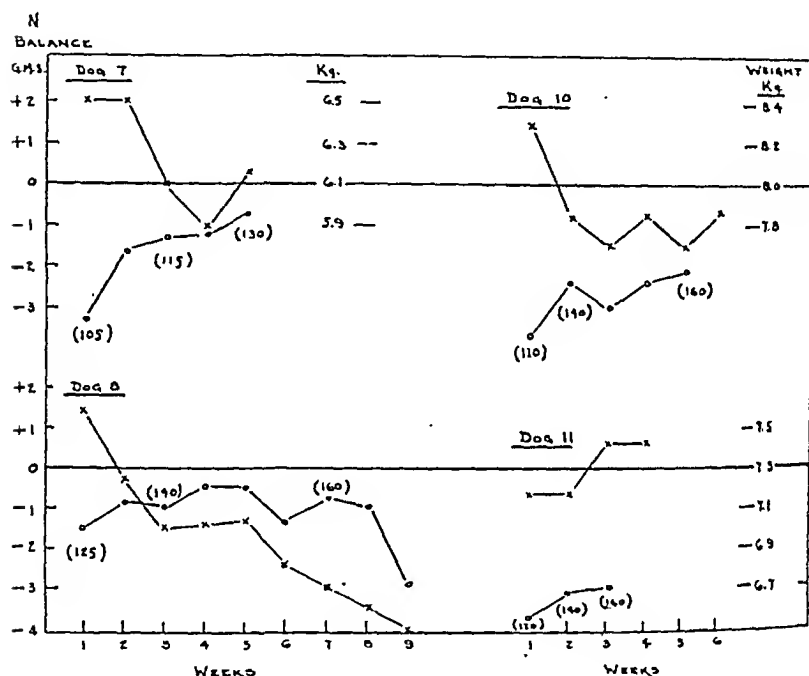


Fig. 1 Weight and nitrogen balance curves for dogs receiving acid hydrolysates of casein. Milligrams nitrogen injected per kilo per day are shown in brackets adjacent to the nitrogen balance curves. Dogs 7 and 8 received casein hydrolysate, 5% unfortified; both dogs died thus terminating the experiment. Dogs 10 and 11 received casein hydrolysate, 5%, with added dextrose, 5%, and cystine, 0.08%. The experiments were terminated to spare the dogs and to get liver biopsy samples.

kilo as the weights of the dogs declined. Thus at the lowest weights of the two dogs, i.e., 5.12 kg for dog 5 and 5.23 kg for dog 3, the input was 170 mg and 160 mg N per kilo, respectively, as against 120 mg N per kilo for both dogs based on their standard weights.

The voluntary food intake averaged about 50 cal. per kilo per day, based on the standard weights of the dogs. Where dextrose was given in conjunction with the hydrolysates, the caloric input in this form ranged from about 3-4.5 cal. per kilo from the smallest to the largest daily injection, i.e., levels

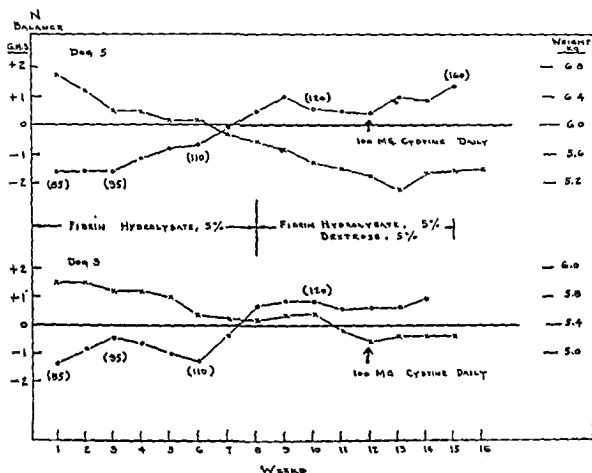


Fig. 2 Weight and nitrogen balance curves for dogs receiving fibrin acid hydrolysates. Milligrams nitrogen injected per kilo per day are shown adjacent to the nitrogen balance curves. Dog 3 was finally sacrificed following a 2-day respiratory infection and was autopsied. Dog 5 was taken off experiment at the same time in order to obtain a liver biopsy sample.

of 110-160 mg N per kilo per day. The added dextrose therefore, accounted for less than 10% of total caloric intake.

Dog 3, autopsied after 14 weeks on fibrin hydrolysate, showed normal kidney, heart, spleen, and adrenals.² The liver of this dog showed a strong, positive fat stain, fatty infiltration, marked cloudy swelling, and cellular vascularization.

²Dr. R. K. Richards performed the autopsy, the liver biopsies, and the microscopic examination of liver slices.

The liver sample obtained from dog 5 by biopsy also showed a strong positive fat stain and fatty infiltration. The cytoplasm was coarsely granular and swollen and contained large vacuoles. The liver biopsy sample from dog 10 after 4 weeks on casein hydrolysate with cystine showed a weak, positive fat stain, fatty infiltration, and some granulation and swelling in the cytoplasm. The sample from dog 11 had negative fat stain, but some cytoplasmic swelling and granulation.

No difficulty was experienced in carrying out the daily injection of the fibrin hydrolysate over the entire period, and no damage to the veins was apparent. A thinning of the hair and scaliness of the skin became evident in both dogs 3 and 5 toward the end of the experiment.

DISCUSSION

Allison and Anderson ('45) derived the relationship between nitrogen balance (NB) and absorbed nitrogen (AN) to yield an indirect evaluation of biological value (BV). The equation is $NB = BV(AN) - EN$; where EN represents endogenous nitrogen excretion. The assumption is made that EN is a constant under ordinary circumstances. When a plot of nitrogen balance is made against absorbed nitrogen, the slope of the line represents the rate of change of nitrogen balance with respect to absorbed nitrogen. This value has been designated as the nitrogen balance index by Allison, Seeley, Brown and Anderson ('46) with reference to the classical concept of biological value as defined by Mitchell ('24, '44).

The present studies offered an opportunity to apply the above principle to derivation of a figure for the nitrogen balance index of fibrin hydrolysate given intravenously. A plot of the nitrogen balance data for dogs 3 and 5 is shown in figure 3. The changing weights of the dogs for each period were used in reducing the weekly values to the basis of milligrams per kilo body weight per dog. Although the points show considerable scatter, it is possible to construct representative lines for each dog. The slopes of the lines as

drawn, representing the nitrogen balance indices, are 0.83 and 0.86 for dogs 3 and 5, respectively. It will be noted that our results are, of necessity, expressed on a per kilo weight basis whereas those of Allison and Anderson ('45) are on a per square meter surface basis, calculated on the changing weights of the dogs.³

The limiting deficiency in fibrin hydrolysates at the low level of input used has not been determined. The fatty infiltration seen in the livers of dogs 3 and 5 is suggestive of

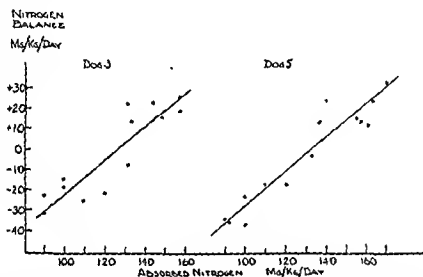


Fig. 3 A plot of nitrogen input vs. nitrogen balance for dogs 3 and 5. The curves are assumed to originate at 0 nitrogen input. The equation describing the curve is, $NB = k(AN) - NE_0$, where NB is nitrogen balance, AN is absorbed (or injected) nitrogen, NE_0 the excretion of nitrogen on a protein free diet (assumed to be a constant), and k the slope of the line. k is equal to that fraction of nitrogen input retained in the body, the biological value, or nitrogen balance index.

methionine deficiency as observed in other species. The slight response to cystine addition in these dogs may also be of significance. Madden et al. ('43) have stressed the importance of methionine for long-continued intravenous nutrition in dogs and have described the more dynamic effects of cystine.

A possible explanation of the continued weight loss in the face of a positive nitrogen balance seen in dogs 3 and 5 may be the shift in liquid balance with a decrease in available body

³ Personal communication from Dr. J. B. Allison.

fluid as the hypoproteinemia regressed. The dogs were hypoproteinemic throughout the study, however, and this condition was not clearly corrected by the low level of nitrogen given. It appears that the level at which a protein material will support nitrogen balance is not always a safe criterion as to the appropriate dietary level of the material for long-term maintenance, especially if equilibrium is approached from the negative side. Weight balance should also be considered as well as the general health of the animal.

The complete failure of unfortified casein hydrolysates to support nitrogen balance at levels up to 160 mg N per kilo per day is apparent from the work with dogs 7, 8, and 12. The inadequacy of casein hydrolysate with added cystine at about 1.6% is indicated from the records of dogs 10 and 11. Analysis for the essential amino acids in casein and fibrin hydrolysates (Frost, Heinsen and Olsen, '46) indicated the cystine contents to be about 0.6 and 2.2% and the methionine contents to be about 2.5 and 1.8%, respectively, on a dry basis.⁴ Thus, in those instances in which cystine was added, the casein hydrolysate was as rich in cystine and richer in methionine than the fibrin hydrolysate.

The superiority of partial acid hydrolysates of fibrin over those of casein has been further demonstrated in rat growth studies in this laboratory.⁵ At 18% of the diet, dried partial acid hydrolysate of fibrin gave growth equal to that obtained with 18% casein, whereas casein hydrolysate produced only negligible growth. The addition of cystine or methionine to casein or dried casein hydrolysate produced a marked growth response in rats whereas addition of these amino acids to fibrin hydrolysate gave only minor stimulation of the growth rate. Risser ('46) previously demonstrated the marked deficiency effect of sulfur amino acids in casein fed orally to dogs. It was also clear from his studies that fibrin contained an adequate complement of cystine and methionine.

⁴ The methionine, cystine, and tryptophane determinations were made by Mr. E. O. Krueger.

⁵ The rat growth experiments were conducted by F. Peirce Dann.

Maintenance of nitrogen balance in intravenous studies with dogs (Risser, Schenck and Frost, '46a, '46b) with casein hydrolysate was accomplished only with a high cysteine supplement and the periods of injection were relatively short as compared with present studies. Kade, Houston, Krauel and Sahyun ('46) maintained dogs in positive nitrogen balance on 214-230 mg N per kilo per day in the form of a complete hydrolysate of casein fortified with 1% dl-tryptophane given intravenously for 1-week periods. They state that this level is near minimal; however, no supplement of sulfur amino acids was made in their studies.

The levels of sulfur amino acids are nearly equalized between the two protein hydrolysates in the present studies, but in view of the earlier results, the casein hydrolysate still appears deficient in sulfur amino acids. The possibility appears, therefore, that the cystine and/or methionine of casein and partial hydrolysates of casein is not completely utilized. This possibility is being studied further.

A basis for difference in utilization of the partial acid hydrolysates of casein and fibrin also resides in the wide difference in tryptophane content, i.e., about 0.55 and 1.1% for casein and fibrin, respectively. Risser, Schenck and Frost ('46b) indicated, however, in short term studies that the relatively low level of tryptophane in the casein hydrolysate is probably not critical. In view of the many variables involved in this type of study exact deductions as to critical levels of the essential amino acids are difficult to make and will require extensive study under a variety of conditions. There are, of course, other bases for difference in utilization between fibrin and casein hydrolysates given intravenously. Chief among these is the factor of peptide utilization about which very little is known.

SUMMARY

Two dogs were maintained for 15 weeks on fibrin hydrolysate (acid hydrolysate) and fibrin hydrolysate plus dextrose intravenously at levels ranging from 85-120 mg

nitrogen per kilo per day. Nitrogen balance became positive when the level was raised to 110 mg nitrogen per kilo, although weight loss continued. The nitrogen balance index for fibrin hydrolysate given intravenously was calculated to be 0.83-0.86.

Partial acid hydrolysates of casein, alone or with an isocaloric addition of dextrose, failed to maintain nitrogen balance when given intravenously in four dogs at levels up to 160 mg nitrogen per kilo per day. Addition of 1.6% cystine on a dry basis failed to improve the casein hydrolysate markedly under the conditions of the experiments.

ACKNOWLEDGMENT

Acknowledgment is gratefully made to Robert T. Olsen and Oscar Walther for technical administration of the hydrolysates and to Mr. H. C. Spruth and Mr. E. B. Vliet for departmental cooperation in making the work possible.

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VITAMIN B COMPLEX STUDIES WITH DIETS DIFFERING IN THE CARBOHYDRATE COMPONENT¹

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ONE FIGURE

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Highly purified diets containing sucrose or dextrin, supplemented with all of the recognized B vitamins except folic acid and biotin, support excellent growth of rats. Such diets are inadequate, however, when poorly absorbed sulfonamides are included and a deficiency syndrome develops that may be overcome by the administration of solubilized liver or folic acid and biotin (Daft et al., '42; Nielsen and Elvehjem, '42; Weleh and Wright, '43; Schweigert et al., '45). It is probable that in the absence of poorly absorbed sulfonamides the requirement of the rat for folic acid and biotin is satisfied by intestinal synthesis. The studies of previous investigators, recently reviewed by Najjar and Barrett ('45), have indicated that bacterial synthesis of the B vitamins in the intestine may be materially influenced by the composition of the diet. Changes in the carbohydrate component of the diet, for example, have been shown to alter both the intestinal synthesis of certain B vitamins and the dietary requirement thereof. The experiments reported in this paper were undertaken in an effort to obtain additional information on the role of the carbohydrate component and the effect of succinylsulfathiazole (SST) in vitamin B synthesis and utilization by the rat.

¹ Presented before the American Institute of Nutrition, March, 1946. (Federation Proceedings, vol. 5, p. 240, 1946).

EXPERIMENTAL

Weanling male albino rats weighing about 40 gm were used. There were four rats in each group that received purified diets containing sucrose, cerelose, dextrin, corn starch, or lactose. Six rats in each group received the same carbohydrate diets containing 2% SST. The diets employed were composed of carbohydrate, 618 gm; casein, 180 gm; salt mixture (Hubbell, Mendel and Wakeman, '37), 40 gm; cellu flour, 40 gm; choline chloride, 1 gm; "Primex," 100 gm; corn oil, 20 gm; A-D-E concentrate (Wright et al., '45), 1 gm; "Menadione," 10 mg; thiamine hydrochloride, 8 mg; riboflavin, 16 mg; nicotinic acid, 40 mg; pyridoxin hydrochloride, 8 mg; calcium pantothenate, 44 mg; p-aminobenzoic acid, 40 mg; and inositol, 216 mg. When succinylsulfathiazole was included in the diet it replaced 2% of the weight of the carbohydrate employed. Records were kept of the appearance and weight changes of all the animals, and, at intervals, of the food consumption. Determinations of the folic acid, biotin, pantothenic acid, nicotinic acid, and riboflavin content of the feces were made at weekly intervals. The pooled samples from all of the rats in each group, collected over a 24-hour period, were divided into two portions. One portion was digested with 2% of its weight of takadiastase in 1% acetate buffer at pH 4.0 under benzene for 24 hours (Cheldelin et al., '42), prior to assay for folic acid (Landy and Dicken, '42), pantothenic acid (Skeggs and Wright, '44), nicotinic acid (Snell and Wright, '41) and riboflavin (Snell and Strong, '39). The other portion was autoclaved in 10 ml of 6 N H_2SO_4 for 1 hour at 120°C. prior to microbiological assay for biotin (Wright and Skeggs, '44). The bacterial flora of the feces were followed at weekly intervals by the dilution-plate count method of Strawinski et al. ('46). White blood cell and differential counts were made at intervals throughout the feeding period on blood obtained by clipping the tail, and at the conclusion of the experiment, when the rats were sacrificed, on blood obtained following decapitation. The livers were removed from all of the animals immediately following decapitation and were divided into two

parts. One part was digested with 2% of its weight of taka-diastase in 20 ml of water under benzene prior to microbiological assay for folic acid, pantothenic acid, nicotinic acid and riboflavin. The other part was autoclaved for 1 hour at 120°C. in 10 ml 6 N H_2SO_4 prior to assay for biotin.

RESULTS

In the absence of SST all of the diets except that containing lactose promoted good to excellent growth and appearance of the rats (fig. 1). The greatest weight gains occurred in rats receiving corn starch, with the least gains in the rats ingesting sucrose. The animals fed lactose developed diarrhea, lost weight, and failed to survive. No attempt has been made to summarize the limited growth data obtained on lactose diets.

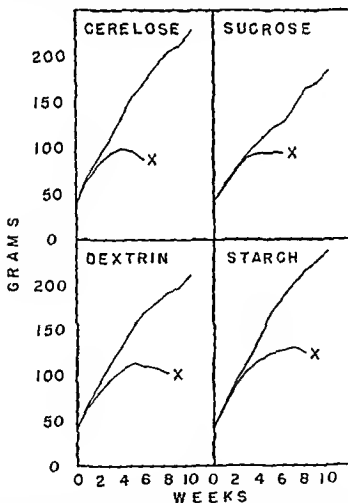


Fig. 1 Growth curves of the rats receiving the various carbohydrate diets. In each instance the upper and lower curves represent, respectively, the results obtained with the basal diet and the corresponding SST-containing diet.

The inclusion of SST in diets containing sucrose, cerelose, dextrin, or corn starch resulted in poor growth and early death. Irrespective of the carbohydrate fed the rats receiving SST developed alopecia, porphyrin-stained whiskers, sore eyes and rough coats.

The food intake studies (table 1) indicated that the poor growth resulting when SST was included in the diet was due

TABLE 1
Summary of food intake data.

CARBOHYDRATE	WEEKS ON TEST	NUMBER OF SAMPLES	FOOD INTAKE			
			No succinylsulfathiazole		2% succinylsulfathiazole	
			Average intake	Range	Average intake	Range
Sucrose	4	12	gm	gm	gm	gm
	5	12	9.4	7-11	6.3	3-10
	7	16	9.8	6-13	5.1	1-5
Dextrin	5	12	10.7	6-15	5.9	0-11
	4	12	10.7	7-14	7.9	6-9
	5	12	11.7	10-15	6.6	4-10
	7	16	11.6	9-15	5.1	2-8
Cerelose	8	12	12.3	10-15	4.8	1-14
	4	12	12.2	10-15	7.3	2-11
	5	12	13.4	8-16	7.3	4-12
Corn starch	7	16	12.9	6-16	6.6 ¹	0-12
	4	12	12.2	9-14	9.6	7-11
	5	12	13.1	10-16	8.8	6-12
	7	16	13.2	11-17	7.9	5-11
	8	12	13.7	11-17	9.3	1-14

¹ Average of 10 determinations.

in part to loss of appetite. The sulfonamide-fed rats generally ate less, and as the deficiency signs became more apparent, certain animals refused all the food offered to them.

The fecal elimination of the various factors, when considered as the amount of each factor excreted per rat in 24 hours, varied with the diet (table 2). On this basis the dextrin-fed rats eliminated appreciably more of all of the factors

TABLE 2¹
Fecal elimination of B vitamins.

DIET	NO. OF DIETS	FOLIC ACID		BIOTIN		PANTOTHENIC ACID		NICOTINIC ACID		RIBOFLAVIN	
		μg/rat/day	μg/gm feces	μg/rat/day	μg/gm feces	μg/rat/day	μg/gm feces	μg/rat/day	μg/gm feces	μg/rat/day	μg/gm feces
Sucrose	9	4.7 (1.5-6.3)	3.9 (2.5-4.6)	0.39 (0.22-0.48)	0.34 (0.22-0.44)	26 (13-31)	23 (18-29)	31 (16-38)	29 (18-43)	18 (9-27)	17 (12-24)
Sucrose + SST	8	0.90 (0.38-1.4)	1.2 (0.6-1.7)	0.32 (0.19-0.43)	0.42 (0.24-0.56)	17 (13-22)	22 (19-29)	29 (19-38)	35 (33-44)	11 (7-16)	14 (10-21)
Dextrin	9	6.7 (4.9-10.0)	4.7 (3.2-6.5)	1.6 (0.95-2.1)	1.2 (0.78-1.24)	71 (54-106)	50 (26-63)	108 (96-124)	78 (55-91)	23 (15-28)	17 (15-22)
Dextrin + SST	9	1.6 (0.86-2.3)	1.3 (1.0-1.6)	1.2 (0.51-1.5)	1.0 (0.30-1.26)	40 (24-63)	32 (26-37)	93 (50-128)	75 (62-95)	28 (13-43)	23 (17-33)
Cerelose	9	4.1 (2.0-6.7)	3.3 (2.2-4.1)	0.62 (0.46-0.85)	0.50 (0.35-0.62)	27 (10-32)	23 (14-34)	27 (21-35)	22 (17-30)	12 (7-21)	10 (7-13)
Cerelose + SST	8	1.5 (0.85-2.3)	1.5 (1.3-2.2)	0.37 (0.24-0.47)	0.40 (0.27-0.51)	20 (11-30)	21 (16-24)	29 (15-47)	30 (24-39)	10 (5-18)	10 (8-13)
Corn starch	9	3.9 (3.1-6.9)	3.2 (2.3-4.0)	0.75 (0.47-1.0)	0.64 (0.52-0.78)	30 (22-38)	25 (19-32)	35 (22-51)	29 (21-31)	11 (6-17)	10 (6-12)
Corn starch + SST	9	1.4 (0.70-2.7)	1.1 (0.7-1.6)	0.44 (0.28-0.61)	0.39 (0.28-0.47)	23 (19-34)	21 (17-26)	32 (27-38)	30 (24-36)	11 (7-14)	9 (7-15)

¹ The figures in parentheses in this and subsequent tables indicate the range in values obtained.

studied than did the animals on the other carbohydrate diets. The elimination of folic acid was markedly lower and that of biotin and pantothenic acid slightly lower when SST was included in the diets. Nicotinic acid and riboflavin elimination was not affected by the drug. However, the food intake studies have shown that less food was consumed by the rats ingesting SST. Since the amount of feces excreted is somewhat dependent on the amount of food consumed, it may be that the growth factors eliminated per gram of fecal material constitute a more accurate basis for a comparison of bacterial synthesis promoted by the various diets. On this basis the synthesis of folic acid per gram of feces was definitely less when SST was included in all of the carbohydrate diets. The decrease in the elimination of biotin and pantothenic acid in most cases, probably was caused, not by decreased synthesis or increased destruction of these factors, but by a diminution in the intestinal bulk, since the incorporation of SST in the diet was accompanied by decreased food intakes. The increased production of the various factors by the dextrin-fed rats would appear to be caused by actual stimulation of intestinal synthesis rather than merely by an increase in the amount of material passing through the intestine.

The variations found in the fecal elimination of the various factors cannot be explained by obvious changes in the intestinal flora. The inclusion of SST, irrespective of the carbohydrate in the diet, resulted in a decrease in the number of coliforms present, but neither the drug nor the carbohydrate exerted any other significant effect on the type or number of organisms found in the feces. That the actual number of organisms present, however, is no criterion of the capacity for synthesis of the B vitamins has been shown by the *in vitro* studies of Miller ('44) who found that, while cultures of *Escherichia coli* may attain equal turbidity (i.e., equal numbers of organisms), those organisms grown in the presence of sulfanilamide produce less folic acid than those grown in a similar medium containing no drug.

CARBOHYDRATE	DAYS ON TEST	NO SUCCINYL-SULFATHIAZOLE				2% SUCCINYL-SULFATHIAZOLE			
		No of defns.	Total white count	Total neutrophils	No. of defns.	Total white count	Total neutrophils	No. of defns.	Total neutrophils
Sucrose	42	4	20,600 (10,300-29,000)	2,350 (1,100-4,000)	6	8,600 (4,400-12,500)	706 (152-2,080)		
	56	4	18,100 (12,200-25,100)	3,860 (2,190-4,750)	4	4,100 (3,000-5,200)	245 (162-354)		
	63	4	8,400 (6,200-13,600)	1,150 (830-1,860)					
Dextrin	42	4	18,700 (10,800-24,000)	2,230 (1,560-3,120)	6	9,800 (4,500-15,800)	630 (316-1,930)		
	63	4	18,300 (14,300-25,900)	2,940 (2,400-3,220)	4	3,300 (1,100-5,100)	172 (32-278)		
	70	4	8,500 (4,300-12,800)	1,060 (610-1,830)					
Cerelese	42	4	17,800 (14,200-20,200)	1,970 (1,300-2,600)	6	8,000 (3,400-13,700)	780 (85-1,890)		
	56	4	20,100 (17,600-23,300)	2,200 (1,820-2,720)	2	3,300 (3,200-3,300)	97 (12-180)		
	70	4	6,800 (5,000-11,500)	790 (450-1,260)					
Corn starch	42	4	16,700 (12,100-21,600)	1,740 (850-2,780)	6	9,000 (4,200-11,900)	671 (85-980)		
	63	4	21,500 (20,300-24,200)	2,970 (1,560-4,500)	5	4,700 (2,100-6,800)	356 (195-945)		
	70	4	8,100 (6,300-8,500)	940 (720-1,140)					

TABLE 4
Hepatic storage of B vitamins.

DIET	DAYS ON TEST	NO OF DET'S.	FOLIC ACID	BIOTIN	PANTOTHENIC ACID	NICOTINIC ACID	RIBOFLAVIN
Sucrose	70	4	$\mu\text{g/gm}$ 0.42 (0.39-0.50)	$\mu\text{g/gm}$ 0.42 (0.31-0.52)	$\mu\text{g/gm}$ 87 (82-96)	$\mu\text{g/gm}$ 178 (161-192)	$\mu\text{g/gm}$ 30 (27-31)
Sucrose + SST	56	4	0.25 (0.18-0.34)	0.28 (0.19-0.40)	55 (42-75)	182 (135-217)	31 (26-39)
Dextrin	70	4	0.55 (0.44-0.81)	0.99 (0.65-1.3)	211 (161-234)	166 (154-175)	38 (29-35)
Dextrin + SST	63	4	0.49 (0.15-0.66)	0.64 (0.55-0.79)	76 (51-103)	160 (120-187)	31 (26-35)
Cerelose	70	4	0.61 (0.38-0.98)	0.72 (0.55-0.81)	115 (98-135)	170 (155-190)	32 (29-38)
Cerelose + SST	56	2	0.26 (0.13-0.39)	0.26 (0.24-0.28)	63 (62-65)	146 (129-163)	26 (25-27)
Corn starch	70	4	0.38 (0.35-0.41)	0.63 (0.46-0.83)	136 (102-159)	179 (158-200)	30 (24-33)
Corn starch + SST	63	4	0.22 (0.17-0.28)	0.30 ¹ (0.17-0.39)	59 ¹ (46-66)	163 ¹ (146-176)	28 (26-32)

¹ Average of 5 determinations.

A summary of the white blood cell data is given in table 3. The leucopenia and granulocytopenia characteristic of sulfonamide-induced folic acid deficiency were found in all of the rats fed SST, irrespective of the type of carbohydrate in the diet. The control rats on each carbohydrate diet had a normal leucocyte picture when the SST-fed rats were sacrificed. The terminal counts on the control groups, however, generally were lower with respect to both total and neutrophil counts than was anticipated. It was felt that the low terminal counts might be indicative of an incipient folic acid deficiency. Kornberg and his associates ('45) have reported that a small percentage of rats ingesting purified diets over a long period of time do develop leucopenia and granulocytopenia that may be alleviated by the administration of folic acid.

It was reported previously (Wright et al., '45) that a level of approximately 0.5 γ or less of folic acid per gram of liver is associated with evidences of a folic acid deficiency in rats. From table 4 it may be seen that in general the rats receiving SST had a hepatic storage characteristic of a folic acid deficiency. The hepatic storage of folic acid in rats ingesting the various carbohydrate diets without drug was approaching the level previously described as critical, confirming the tendency toward a folic acid deficiency suggested by the low leucocyte counts. The liver storage of biotin and pantothenic acid was depressed by the inclusion of SST in all the carbohydrate diets. Nicotinic acid and riboflavin were found in the liver in normal amounts regardless of the carbohydrate or drug in the diet. The storage of pantothenic acid in the livers of rats fed the control dextrin diet was remarkably high. Because of the large amounts of folic acid, biotin, nicotinic acid and pantothenic acid eliminated in the feces of the dextrin-fed rats, an increased liver storage of all of these factors might have been expected. Actually pantothenic acid was the only factor to be stored in unusually large amounts.

DISCUSSION

These studies have demonstrated that the type of dietary carbohydrate used in highly purified diets is of little significance in the production by SST of a combined folic acid, biotin and, frequently, pantothenic acid deficiency. The study made of food consumption and fecal elimination of the various factors has clarified, to a large extent, the role of SST in relation to the intestinal synthesis of essential factors. The *in vitro* studies of Miller ('44) have shown that the presence of subbacteriostatic amounts of sulfanilamide in cultures of *Escherichia coli* depresses the ability of the organism to synthesize folic acid. This principle apparently applies *in vivo*. The administration of SST to the rat does not alter appreciably the intestinal flora, except for a reduction in the number of coliforms, but does act directly to decrease the synthesis of folic acid. Animals subsisting on purified diets that do not contain poorly absorbed sulfonamides have at best a marginal hepatic storage of folic acid and biotin. Since the only source of these factors in such diets is that produced by intestinal synthesis, the action of SST is partially to block off the source of supply and the rats develop a folic acid deficiency. As the animals become deficient, food intakes tend to diminish. The data have shown that the amount of biotin produced per gram of feces generally is not altered by the administration of the drug. Through decreased food intake, however, the intestinal bulk is diminished and consequently less biotin becomes available to the animal for growth. Depletion of the small amount of biotin stored in the tissues ensues. At present, the frequent instances of pantothenic acid deficiency are more difficult to explain. Although decreased food consumption as a result of the combined folic acid and biotin deficiencies is undoubtedly a factor, other lines of evidence indicate that this is not a complete explanation.

SUMMARY

1. A study has been made of the growth, food consumption, intestinal flora, leucocyte picture, fecal elimination and

hepatic storage of folic acid, biotin, pantothenic acid, nicotinic acid and riboflavin of rats receiving highly purified diets containing glucose, sucrose, lactose, dextrin or corn starch as the carbohydrate component, with and without the addition of succinylsulfathiazole (SST).

2. The inclusion of SST in highly purified diets irrespective of the carbohydrate component is effective in producing a combined folic acid and biotin deficiency.

3. The intestinal synthesis of folic acid, and consequently the amount available to the animals, is depressed directly by the administration of SST.

4. The feeding of purified diets containing no drug promotes the synthesis of sub-optimal amounts of folic acid since prolonged feeding of such diets may result in hepatic stores of folic acid indicative of an incipient deficiency.

5. In the absence of SST, the carbohydrate component of the diet, as in the case of dextrin, may influence the synthesis of certain B vitamins in the intestine of the rat, and indirectly the tissue storage of pantothenic acid.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

I. BY THE GROWING ALBINO RAT¹

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In a recent number of this journal Forbes, Swift, Elliott and James ('46) published results of experiments showing that with growing rats on diets containing 2, 5, 10 and 30% of fat, respectively, and so compounded and fed as to supply identical quantities of energy and of protein, the gains in live weight, the digestibility of nitrogen, and the retention of nitrogen and energy were in the order of the increasing fat contents of the diets; and that with mature rats on the same diets as above specified the energy expense of utilization (dynamic effects) of these diets diminished in the order of the increasing fat contents of the diets.

In the conduct of these experiments it was the intent of the authors to compound the diets in such manner as to supply all nutrients required for the most efficient utilization of food energy; but it is conceivable that the vitamin requirements were not completely satisfied.

It was decided, therefore, to repeat these experiments with three of the diets, those containing 2, 10 and 30% of fat, with large increases in ten of the vitamins.

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EXPERIMENTAL

In the repetition of these experiments the diets with both the growing and the mature rats were composed as is shown in table 1, the results with growing rats being given in the present paper and those with mature rats in the paper which follows. Though the present paper presents results of the experiments of 1945-46 alone, the amounts of the vitamins supplied as supplements to the diets of this year, and of the preceding year as well, are given for comparison in table 2.

In both years' experiments it was assumed that the requirements of the rats for essential fatty acids were satisfied by the corn oil, and that the requirements for vitamin D, biotin and folic acid were satisfied by the yeast component of the diets.

The quantitative basis for increasing the intake of vitamins in the experiments of 1945-46 as compared with those of 1944-45 was largely empirical. The increase in the amount of choline was suggested by C. A. Elvehjem (letter of September 22, 1945); and H. E. Longenecker suggested (letter of September 27, 1945) that the amounts of pyridoxine and of pantothenic acid provided in the experiments of 1944-45 might have been at borderline levels. The amounts by which the allowances of the other vitamins were increased, however, were determined in a purely arbitrary manner.

The method of computation of the makeup of the diets as required to supply fat at three levels, with protein constant, and with carbohydrate varying reversely as the fat, in order to provide a constant energy intake, will be given.

Referring to table 1, it will be noted that with the salt mixture and the corn oil as constants, the three levels of fat intake were provided by the introduction of lard into diets no. 2 and 3. The protein and the carbohydrate mixtures were arbitrarily devised; and the amounts supplied of each, in order to provide protein at a constant level, and carbohydrate in the quantities required to maintain a constant energy intake in the three diets, were computed by an algebraic process which will be explained. The nitrogen content of the protein

mixture was 11.05%; and the energy values of the dietary components, as determined by the bomb calorimeter, were as follows: protein mixture 4825 cal., carbohydrate mixture 3697 cal., corn oil 9382 cal., and lard 9431 cal., per gm. These values are the average of duplicate or triplicate determinations which differed among themselves by less than 1%.

Diet no. 1 was arbitrarily made up to contain 4% salt mixture, 2% corn oil, and 22% protein. Using this combination,

TABLE 1
General composition of diets.

COMPONENTS	DIET NO. 1	DIET NO. 2	DIET NO. 3
	%	%	%
Salt mixture ¹	4.00	4.00	4.00
Corn oil ²	2.00	2.00	2.00
Protein mixture ³	31.86	35.84	45.82
Carbohydrate mixture ⁴	62.14	50.16	20.18
Lard ⁵	0.00	8.00	28.00
Cal. per gm	4.02	4.526	5.785
Isocaloric factors	1.0000	0.8887	0.6953

¹ U.S.P. XII no. 2.

² The energy of the corn oil was 9.382 Cal. per gm.

³ Casein 70%, skim milk powder 15%, irradiated yeast 10%, and brewer's yeast 5%. Mixture contained 11.05% N, and 4.825 Cal. per gm.

⁴ Corn starch 34%, sucrose 33%, dextrin 12%, and dextrose (cerelose) 21%. Mixture contained 3.697 Cal. per gm.

⁵ The energy of the lard was 9.431 Cal. per gm.

TABLE 2
Vitamins added per kg of diet 1 and to isocaloric quantities of other diets in experiments of 1944-45 and 1945-46.

	1944-1945	1945-1946		1944-1945	1945-1946
	mg	mg		mg	mg
Carotene	6.0	40	Choline chloride	400	2000
Thiamine hydrochloride	5.0	20	Alpha-tocopherol	100	200
Riboflavin	5.0	20	Inositol	2000	2000
Pyridoxine hydrochloride	6.25	20	Para-aminobenzoic acid	95	200
Niacin	6.25	20	2-methyl-1,4 naphthoquinone	2.5	6
Calcium pantothenate	50	100			

the theoretical amount of protein mixture required per 100 gm of the diet was found to be 31.8552 gm, leaving 62.1448 gm to be provided by the carbohydrate mixture, the energy value of the whole being 4022 cal. per gm, and the ratio of the energy value of the protein mixture to the energy value of the complete diet being as 0.3821:1. It then remained to compute the other diets in such manner as to contain 10% and 30% of fat, respectively, and to provide protein and energy in the same quantities as supplied by diet no. 1. To compute the 10%-fat diet, the composition of diet no. 1 was modified by adding 8% of lard to the 2% of corn oil and 4% of salt mixture, leaving 86% of the diet to be provided by the protein and carbohydrate mixtures.

If x represented the percentage of protein mixture per 100 gm of diet no. 2, and $86-x$ represented the percentage of carbohydrate mixture, then

$$\frac{4825 x}{4825 x + (86-x) 3697 + 2 (9382) + 8 (9431)} = 0.3821$$

This gave a percentage of 35.84 for x , and left a percentage of 50.16 for the carbohydrate mixture. These factors were set up on the calculating machine. When the rations were made up (3 times during the experiment), the amounts of all five components listed in table 1 were weighed to an accuracy of 1 gm.

The energy value of this diet was 4526 cal. per gm, and its protein content was 24.76%. The ratio of the energy of equal quantities of diet no. 1 to diet no. 2, as well as the ratio of their protein contents was as 1 to 0.889. Therefore, for every gm of diet no. 1 there was fed 0.889 gm of diet no. 2, which supplied equal amounts of protein and energy—the only difference being in the sources of the nonprotein energy. The makeup of the 30%-fat diet was computed by the same procedure; and the results of these computations are given in table 3. Daily feeds were weighed on a chainomatic balance sensitive to 0.1 mg.

Since the diets of different fat contents also differed in their carbohydrate contents it is necessary to stress the fact that it would be as logical to relate the differences in their nutritive value to the differences in carbohydrate as to the differences in fat. However, the results of this experiment are discussed in relation to the fat contents of the diets for the sake of convenience, and because of interest in the physiological effects and dietetic relationships of fat.

As in the earlier experiment with growing rats, the subjects in the present experiment were allowed normal freedom of movement. The method of experimentation provided for the computation of a single measurement of the heat production for a 70-day period as the gross energy of the feed minus

TABLE 3

Constituents of diets of different fat and different carbohydrate contents which supply the same quantities of protein and energy.

DIET NO.	APPROXIMATELY ISOCALORIC QUANTITIES OF DIETS	PROTEIN		FAT		CARBOHYDRATE		ENERGY	
		gm	%	gm	%	gm	%	cal / gm	cal
1	10.00	22.00	2.20	2.00	.20	65.26	6.53	4022	40.22
2	8.89	24.76	2.20	10.00	.89	54.91	4.88	4526	40.24
3	6.95	31.64	2.20	30.00	2.09	28.68	1.99	5785	40.21

the energy of the excreta and of the body gain. The experiments with mature rats as subjects, with voluntary activity excluded by close confinement in respiration chambers, were conducted by the open-circuit Haldane procedure, the heat increment or dynamic effect of the diets being determined as the difference in the quantities of heat produced from a basal quantity of food sufficient for maintenance and a larger quantity of the same diet.

The experiment with growing rats involved thirty-six male animals, three rats being taken from each of twelve litters. In each group of three litter mates, each rat received one of the three diets. The animals were 24 to 27 days old at the

beginning of the experimental feeding and 70 days older when they were killed for analysis at the end of the experiment. The food eaten during the 70 days was 773 gm of diet no. 1, and isocaloric quantities of diets no. 2 and 3.

During this test the daily urines were made up to 250 ml, from which a 25 ml aliquot was put into a 2.5 liter bottle in which the urine was preserved with sulphuric acid, and the feces, as collected daily, were dried in the air at room temperature. The energy of the urine was taken at 8.6 ± 0.05 Cal. per gm N. This factor was derived from comparable experiments of the previous year in which similar diets were fed,

TABLE 4

Average liveweights of rats during 10 weeks on isocaloric quantities of diets containing 2, 10 and 30%, respectively, of fat.

DIET NO.	FAT CON- TENTS OF DIET	INITIAL BODY WEIGHT	WEEK NUMBER									
			1	2	3	4	5	6	7	8	9	10
	%	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
1	2	42	59	88	118	145	173	199	224	245	262	274
2	10	42	59	87	117	144	174	200	227	249	266	278
3	30	42	58	87	116	142	171	200	227	249	268	280

and the energy of the urine was determined by the bomb calorimeter.

The average weekly liveweights recorded in table 4 indicate a conceivable advantage in favor of the 2%-fat diet as compared with the 30%-fat diet of 1 gm in the first week, increasing to 3 gm in the fourth, and to 2 gm in the fifth week. Between the fifth and sixth weeks, however, this hypothetical advantage in favor of the low-fat diet disappeared, the weights for the sixth to the tenth weeks indicating an advantage in favor of the 30%-fat diet as compared with the 2%-fat diet of 1, 3, 4, 6 and 6 gm, respectively.

The computed odds that the average liveweight of the rats on the 30%-fat diet were significantly higher than the weight of the rats on the 2%-fat diet were only 11 to 1. The observed differences in average liveweight of the rats which

received the different diets, therefore, were not statistically significant.

It is shown in table 5 that the body gains of nitrogen of the rats diminished, and the gains of fat increased, in the order of the increasing fat contents of the diets, the odds of significance between the 2%- and the 30%-fat diets being 15:1 for the nitrogen retention and 151:1 for the fat gains.

On the other hand, in the earlier experiment there were significant increases in nitrogen retention in the order of the increases in the fat contents of the diets.

TABLE 5

Average amounts of food eaten during 70 days; and initial and final nitrogen and fat contents of rat bodies.

FAT CONTENTS OF DIETS	FOOD EATEN	INTAKE OF NITROGEN	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	gm	gm	gm	gm	gm	gm
2	780.0	27.46	1.02	8.97	3.23	29.76
10	693.2	27.46	1.02	8.83	3.25	34.36
36	542.3	27.46	1.02	8.67	3.24	39.66

It is unnecessary to attempt a fundamental interpretation of this difference in evidence relating to nitrogen utilization, first because it was not technically positive, and second because the relative proportions of protein, carbohydrate and fat catabolized may vary considerably, as influenced by superficial environmental conditions, without such variation being of fundamental significance.

It is shown in table 6 that the urine nitrogen increased, and the fecal nitrogen, as well as the nitrogen retained, decreased, without exception, in the order of the increase in the fat contents of the diets, but the significance of these observations is not to be stressed — for the reasons given above.

It is noteworthy that the nitrogen recovery during the feeding experiment, as indicated by the quantity of nitrogen in the excreta, plus the quantity of nitrogen retained (as deter-

mined by body analysis), divided by the quantity of the nitrogen intake, was 99% or more in each case.

In table 7 is given the partition of the average daily intake of food energy per rat during 70 days.

The decrease in carbohydrate intake compensating for the increasing fat content of the diets was without positive effect

TABLE 6

Partition of average nitrogen intake per rat during 70 days.

FAT CONTENT OF DIETS	NITROGEN				
	Intake	Output in		Retained ¹	Retained ²
		Urine	Feces		
%	gm	gm	gm	gm	gm
2	27.46	17.36	1.89	8.21	7.95
10	27.46	17.61	1.76	8.09	7.81
30	27.46	18.03	1.53	7.90	7.65

¹ Nitrogen in the food minus nitrogen in the excreta.

² Nitrogen in the bodies of the rats which were fed for 70 days minus the nitrogen in the bodies of the control group.

TABLE 7

Partition of average daily intake of food energy per rat during 70 days.

FAT CONTENT OF DIETS	GROSS ENERGY INTAKE	ENERGY INTAKE				ENERGY OUTPUT			ENERGY RE- TAINED
		Protein	Carbo- hydrate	Fat	Metabo- lizable	In feces	In urine	As heat	
%	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.
2	3137	978	2011	148	2872	116	149	2333	539
10	3137	978	1501	658	2844	142	151	2266	578
30	3137	978	614	1545	2815	167	155	2195	620

on the metabolizable energy of the diet. The three factors of energy outgo, however, present a highly significant picture of the effect of food fat on the efficiency of utilization of food energy. Thus, in spite of marked increase in the energy of the feces, and the nonsignificant increase in the energy of the urine, there was a definite decrease in the heat production

(odds 1999:1), and an increase in energy retention (odds 98:1), in the order of the increasing fat contents of the diets.

In the light of present knowledge and evidence, therefore, fat confers efficiency of utilization of food energy for the growing albino rat.

The evidence to this effect was more positive in the present experiment, with large increases in the intake of ten of the vitamins, than in the earlier work from this laboratory, to which the authors have referred, in which a lower level of vitamin intake prevailed.

SUMMARY

A 70-day metabolism and body analysis experiment was conducted to determine the effects of differences in the fat content of diets, with large increases in the intake of ten of the vitamins, as compared with earlier experiments, on the utilization of food energy and protein by growing albino rats.

The subjects were three groups of twelve weanling males, each group containing one rat from each of the same twelve litters.

A comparison was made between three diets containing 2, 10 and 30% of fat, respectively, these diets being so compounded and fed as to supply to each rat of a litter-three, and therefore to each group of 12, the same quantities of energy, protein, and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, and of the heat production for 70 days as the energy of the food minus the energy of the excreta and of the body gain.

The statistically significant results were body gains of fat and energy, and decrease in the heat production, in the order of the increasing fat contents of the diets.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

II. BY THE MATURE ALBINO RAT ¹

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This experiment was a repetition of the greater part of an earlier study by Forbes, Swift, Elliott and James ('46), with greatly increased intake of ten of the vitamins, for the purpose of learning whether the results observed in the former experiment, also with mature rats, were determined to any extent by an intake of less than optimum amounts of these nutrients. The specific object was to study the energy and nitrogen metabolism of isocaloric quantities of diets containing 2, 10 and 30% of fat, respectively, each supplying the same quantity of protein, and each fed at two levels of intake.

For convenience, the two planes of nutrition providing for the relation of the difference in food to the difference in heat production were designated "maintenance" and "super-maintenance," the energy of the higher plane of intake being 45.45% greater than that of the lower plane.

As a matter of fact, however, the exact maintenance requirements of the rats were not determined in the respiration experiments since the metabolizable energy was determined while the animals were free to move about, and the heat production was determined with voluntary activity excluded.

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The general experimental routine was exactly the same as in the earlier study referred to.

The subjects were three groups of twelve essentially mature male albino rats about 6 months old. These three groups of rats were born at intervals of time which made all three groups equal in age when used in the respiration experiments.

The diets were composed as shown in table 1 of the foregoing paper on growing rats (page 399). As shown in table 1 of the present paper, the rats on the 2%-fat diet received 11 and 16 gm each of food at the two planes of nutrition,

TABLE 1

Percentage contents of diets and daily intake of protein, carbohydrate, fat and energy.

DIET NO.	PLANE OF NUTRITION	TOTAL FOOD INTAKE	PROTEIN		CARBOHYDRATE		FAT		ENERGY
			gm	%	gm	%	gm	%	
1	Maintenance	11.00	22.00	2.42	65.26	7.18	2.00	0.22	44.24
2	Maintenance	9.78	24.75	2.42	54.92	5.37	10.00	0.98	44.24
3	Maintenance	7.65	31.63	2.42	28.69	2.19	30.00	2.30	44.24
1	Supermaintenance	16.00	22.00	3.52	65.26	10.44	2.00	0.32	64.35
2	Supermaintenance	14.22	24.75	3.52	54.92	7.81	10.00	1.42	64.35
3	Supermaintenance	11.12	31.63	3.52	28.69	3.19	30.00	3.34	64.35

whereas the rats on the 10%-fat and the 30%-fat diets received smaller quantities of food which contained the same quantities of gross energy and of protein as supplied by the 2%-fat diet.

The schedule of experimentation, given in table 2, was so arranged for each rat that the 2-day maintenance respiration period began immediately after the 8-day maintenance excreta collection period. Then followed (a) 5½ days' preparatory feeding on the supermaintenance plane, (b) the 2-day respiration period on supermaintenance feeding, and (c) the 8-day excreta collection period on supermaintenance feeding—the object of this arrangement being to have the respiration

periods at the two planes of nutrition as close together, in point of time, as practicable.

The digestibility of the food nitrogen (table 3) was essentially the same at both planes of food intake, such difference as existed being an increase in the order of the increase in

TABLE 2
Schedule of experimentation.

EAT NO.	PLANE OF NUTRITION	FAT CONTENT OF DIET	DAILY ENERGY INTAKE	AGE OF RATS	AVE. WEIGHT OF RATS	DATES OF RESPIRATION MEASUREMENTS
		%	Cal.	days	gm	
1-12	Maintenance	2	44.24	169	311	Jan. 28-Feb. 2
1-12	Supermaintenance	2	64.35	176	335	Feb. 4-Feb. 9
13-24	Maintenance	10	44.24	169	310	Mar. 4-Mar. 9
13-24	Supermaintenance	10	64.35	176	335	Mar. 11-Mar. 16
25-36	Maintenance	30	44.24	169	326	Apr. 1-Apr. 6
25-36	Supermaintenance	30	64.35	176	350	Apr. 8-Apr. 13

TABLE 3
Utilization of daily nitrogen.

DIET NO.	PLANE OF NUTRITION	FAT CONTENT OF DIET	NITRO- GEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION	
		%	mg	mg	% of intake	mg	% of intake	mg	% of intake
1	Maintenance	2	387	356	92.0	360	93.0	—4	—1.0
2	Maintenance	10	387	358	92.5	356	92.0	2	0.5
3	Maintenance	30	387	360	93.0	375	96.9	—15	—3.9
1	Supermaintenance	2	563	518	92.0	445	79.0	73	13.0
2	Supermaintenance	10	563	522	92.7	457	81.2	65	11.5
3	Supermaintenance	30	563	522	92.7	475	84.4	47	8.3

the fat content of the diets. At the higher plane of food intake the nitrogen retention decreased in the order of the increasing fat content of the diets, the odds that the retention from the 2%-fat diet was greater than from the 30%-fat diet being more than 10,000 to 1. This fact is not explained by any of the experimental observations made; but in view of the proba-

bility that the composition of the diets affected the synthetic activities of the alimentary microorganisms, it is not surprising that the reasons for some of the facts observed are not in evidence.

Referring to table 4—at both planes of energy intake there were, in the order of the increasing fat content of the diets, barely perceptible increases in the energy of the daily urine; definite increases in the fecal energy; definite decreases

TABLE 4
Intake and partition of daily food energy.

DIET NO.	PLANE OF NUTRITION	INTAKE	FECES	URINE	METABOLIZABLE ENERGY		HEAT PRODUCTION		HEAT PRODUCTION DIVIDED BY METABOLIZABLE ENERGY
		Cal.	Cal.	Cal.	Cal.	Coeff. var. %	Cal.	Coeff. var. %	
1	Maintenance	44.24	1.99	3.07	39.18	0.31	30.37	6.92	0.78
2	Maintenance	44.24	2.27	3.08	38.89	0.59	29.33	5.96	0.75
3	Maintenance	44.24	2.99	3.11	38.14	0.84	27.93	5.19	0.73
1	Super-maintenance	64.35	3.82	4.37	57.16	0.41	36.53	2.80	0.64
2	Super-maintenance	64.35	3.32	4.41	56.62	0.46	34.52	5.14	0.61
3	Super-maintenance	64.35	4.57	4.43	55.35	1.00	31.22	3.03	0.56

in the metabolizable energy; and decreases in the heat production which were definitely more extensive than the decreases in metabolizable energy.

The values for heat production divided by metabolizable energy at the lower plane of nutrition were sufficiently less than unity to indicate that the energy intake at this lower plane of nutrition was well above the maintenance requirement including normal activity. That the values for heat production divided by metabolizable energy diminished in the order of the increase in per cent of fat in the diets signifies that the fat component of the diets conferred efficiency of utilization of their metabolizable energy.

At the higher plane of nutrition the computed odds that the metabolizable energy of the 10%-fat diet was more efficiently utilized than that of the 2%-fat diet were 131 to 1; and the odds that the metabolizable energy of the 30%-fat diet was more efficiently utilized than that of the 2%-fat diet and of the 10%-fat diet were millions to one.

The quantities of heat, and the differences in the amounts of heat, as well as the sources of the heat produced from each diet, are given in table 5. The total heat production at both

TABLE 5

Quantities, increments, and sources of average daily heat production.

DIET NO.	PLANE OF NUTRITION	TOTAL HEAT AND HEAT IN- CREMENTS	NON- PROTEIN R. Q.	SOURCES OF HEAT PRODUCTION			
				Protein	Carbo- hydrate	Fat	Fat Syn- thesis
		<i>Cal</i>		<i>Cal</i>	<i>Cal</i>	<i>Cal</i>	<i>Cal</i>
1	Supermaintenance	30.53	1.20	11.80	23.70	0.00	1.03
1	Maintenance	30.37	1.10	9.54	20.43	0.00	0.40
1	Heat increment	6.16		2.26	3.27		0.63
2	Supermaintenance	34.52	1.09	12.12	21.98	0.00	0.42
2	Maintenance	29.33	.97	9.44	18.00	1.89	0.00
2	Heat increment	5.19		2.68	3.98		0.43
3	Supermaintenance	31.22	.85	12.59	9.45	9.18	0.00
3	Maintenance	27.93	.80	9.93	6.01	11.99	0.00
3	Heat increment	3.29		2.66	3.43		0.00

planes of nutrition, as well as the heat increment for each diet, diminished markedly in the order of the increasing fat contents of the diets.

The conditions of experimentation determined that the heat increments represented the efficiency of utilization of food at a plane of nutrition above maintenance, with voluntary activity excluded, by animals nearing maturity but still able to store considerable amounts of nutriment. The proportions of the heat increments derived from carbohydrate and protein from each diet were similar, but not identical, and these values

appear not to have been affected by the diets in regular order.

With reference to the sources of the total heat production—the greatest difference observed was the decrease in heat from carbohydrate at both planes of nutrition, in the order of the increasing fat content of the diets. Similarly, there was a slight increase in heat production from protein, with one departure from the regular order. No fat was catabolized from the 2%-fat diet, while much heat was produced from fat from the 30%-fat diet. Heat production from fat synthesis occurred in the three periods in which the carbohydrate intake was highest, and ranged from 1.3 to 2.8% of the total heat production.

The results of this experiment, in which the intake of the vitamins was purposely excessive, were in general agreement with the earlier experiment, in which the vitamin intake was not so large, especially in that the heat production at both planes of food intake, and also the heat increment of the diets, decreased in the order of their increasing fat contents.

These findings, together with the demonstration by Forbes, Swift, Marcy and Davenport ('44) that the dynamic effects of isocaloric diets decrease in the order of their increasing protein contents, seem harmonious with the fact that protein and fat are the main components of the animal body.

SUMMARY

Respiration experiments were conducted by the open-circuit Haldane respiratory quotient procedure, with thirty-six mature albino rats as subjects, to investigate the energy expense of utilization (heat increment) of three complete diets differing in fat content and supplying ten vitamins in much larger quantities than in earlier experiments.

The heat increments were measured as the difference in heat production from maintenance and supermaintenance diets containing 2, 10 and 30% of fat, respectively, so compounded and fed as to supply equal quantities of gross energy, protein, and vitamins.

The fat content of the diets had little effect on nitrogen utilization, but with the supermaintenance food intake there was slight increase in urinary nitrogen and decrease in nitrogen retention in the order of the increasing fat content of the diets. There were slight decreases in metabolizable energy and larger decreases in heat production in the order of the increasing fat contents of the diets, the fat component, therefore, conferring economy of utilization of food energy.

The heat increments of the dietary supplements containing 2, 10 and 30% of fat, respectively, representing the use of food above maintenance, with voluntary activity excluded, were equivalent to 31, 26 and 16%, respectively, of their gross energy.

The decreasing energy expense of utilization of the isocaloric intake of the diets in the order of their increasing fat contents was due mainly to decreasing heat from the catabolism of carbohydrates. No fat was catabolized from the 2%-fat diet, while much heat was produced from fat in the 30%-fat diet.

Heat production from fat synthesis occurred in the three periods in which the carbohydrate intake was highest.

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DENTAL CARIES IN THE COTTON RAT

VIII. FURTHER STUDIES ON THE DIETARY EFFECTS OF CARBOHYDRATE, PROTEIN AND FAT ON THE INCIDENCE AND EXTENT OF CARIOUS LESIONS ¹

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Previous studies from this laboratory (Shaw et al., '44 and Schweigert et al., '45b, '46) have shown that the incidence and extent of tooth decay in the cotton rat were markedly influenced by the kind of carbohydrate in the diet. Specifically, ingestion of sucrose or other soluble sugars resulted in a high caries index, while few carious lesions developed on dextrin or stock rations. With diets in which lard or casein had been isocalorically substituted for sucrose, a low caries index was found, and animals fed milk as the sole diet were free of carious lesions (Schweigert et al., '46). It was therefore of interest to study the effects of carbohydrates other than sucrose in diets containing added lard or increased amounts of protein.

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We are indebted to Merck and Company, Rahway, New Jersey, for the crystalline vitamins; to Abbott Laboratories, Chicago, for the halibut liver oil; and to Wilson and Company, Chicago, for the 1 : 20 liver extract and dog food used in this study.

²Now at the Harvard School of Dental Medicine.

EXPERIMENTAL

Weanling cotton rats (15-25 gm) from our stock colony were used in these experiments. The animals were kept on experiment for 14 weeks (Shaw et al., '44) and the growth rate in grams per week was recorded as the growth of males as pointed out by Schweigert et al. ('45b). Since a strain variation in caries susceptibility has been observed (Schweigert et al., '45a) animals from each litter were equally distributed among all experimental groups and the control group fed the sucrose basal ration. At the end of the experimental period, the animals were sacrificed and the incidence and extent of the carious lesions were determined by the method of Shaw et al. ('44).

The rations were prepared at weekly intervals and fed ad libitum. The composition of the rations is shown in table 1.

TABLE 1
Composition of rations.¹

CONSTITUENT (PARTS OF THE RATION)	SUCROSE	LACTOSE	DEXTRIN	STARCH	GLUCOSE	DEXTRI- NALTOSE	CASEIN	CORN OIL	SALTS IV	LARD
<i>Ration no.</i>										
802	67	24	5	4	.
817	67	24	5	4	.
818	67	24	5	4	..
819	33.5	33.5	24	5	4	..
833	...	16.75	50.25	24	5	4	..
825	16.75	.	50.25	24	5	4	.
821	44.5	24	5	4	10
826	...	44.5	24	5	4	10
827	22.25	22.25	24	5	4	10
828	44.5	24	5	4	10
829	44.5	24	5	4	10
830	22.25	22.25	24	5	4	10
831	32.5	36	5	4	10
832	22.25	.	22.25	24	5	4	10

¹ Adequate quantities of the B vitamins were provided (McIntire, Schweigert and Elvehjem, '44).

When lard or additional protein was included in the ration, 1 gm of the former was added at the expense of 2.25 gm of carbohydrate, and 1 gm of protein replaced 1 gm of the carbohydrate.³ Adequate quantities of the B vitamins were provided (McIntire et al., '44) and each rat received 1 drop of halibut liver oil per week. Four per cent of 1 : 20 liver extract was added to each diet at the expense of the entire ration.

RESULTS AND DISCUSSION

The growth rates of all animals for the first 6 weeks and the total 14 weeks on experiment are shown in tables 2 and 3.

TABLE 2

Effects of various carbohydrates on the incidence and extent of carious lesions.¹

RATION FED ²	NO. OF ANIMALS	RATE OF GROWTH		AVE. INCIDENCE OF CARIOUS LESIONS	AVE. EXTENT OF CARIOUS LESIONS
		First 6 weeks	Total 14 weeks		
		<i>gm/week</i>			
802	7	10.2	8.2	23	70 +
817 (starch)	6	6.6	4.6	2	7 +
802	7	9.4	6.5	32	97 +
819 ($\frac{1}{2}$ dextrin, $\frac{1}{2}$ lactose)	7	10.1	6.5	23	63 +
818 (dextrin)	3	9.8	7.0	1	2 +
802	6	10.6	7.6	33	94 +
833 ($\frac{1}{2}$ dextrin, $\frac{1}{2}$ lactose)	4	8.8	7.0	18	46 +
802	4	13.1	8.6	25	57 +
825 ($\frac{1}{2}$ dextrin, $\frac{1}{2}$ sucrose)	4	12.3	7.8	13	19 +

¹ For a detailed description of the method of evaluating the incidence and extent of the lesion see Shaw et al. ('44).

² 4% of 1 : 20 liver extract was added to all diets at the expense of the entire ration. The proportions of the carbohydrate portion of the diet are given in parentheses.

The ingestion of rations in which starch or a combination of lactose and lard had been substituted for sucrose (rations 817 and 826, respectively) resulted in a somewhat slower rate of growth, while the growth rates obtained with the other

³ Glucose was fed as cerelese, a commercial glucose monohydrate. The water of crystallization was taken into account in preparing the rations.

rations were not appreciably different from those of animals fed the sucrose basal. In previous work from this laboratory high lactose diets were found to promote slower growth (Schweigert et al., '45b).

TABLE 3

Effects of various carbohydrates and of the level of fat, carbohydrate, and protein on the incidence and extent of carious lesions.

RATION FED ¹	NO. OF ANIMALS	RATE OF GROWTH		AVE. INCIDENCE OF CARIOUS LESIONS	AVE. EXTENT OF CARIOUS LESIONS
		First 6 weeks	Total 14 weeks		
		<i>gm/week</i>			
802 (sucrose basal)	10	11.0	8.3	28	74 +
821 (sucrose, lard)	7	11.9	8.3	17	42 +
827 ($\frac{1}{2}$ sucrose, $\frac{1}{2}$ lactose, lard)	5	9.1	6.7	14	26 +
826 (lactose, lard)	1 ²	6.8	3.0	10	24 +
802 (sucrose basal)	3	10.3	7.2	22	67 +
828 (dextri-maltose, lard)	4	12.3	8.4	7	12 +
829 (glucose, lard)	4	9.5	7.1	9	16 +
802 (sucrose basal)	5	10.2	6.8	35	101 +
821 (sucrose, lard)	5	10.5	6.6	16	32 +
828 (dextri-maltose, lard)	3	9.2	6.5	26	55 +
829 (glucose, lard)	4	9.0	5.6	24	47 +
802 (sucrose basal)	6	11.4	8.2	31	82 +
821 (sucrose, lard)	3	13.3	10.4	15	32 +
830 ($\frac{1}{2}$ sucrose, $\frac{1}{2}$ dextri-maltose, lard)	7	11.8	8.3	18	34 +
832 ($\frac{1}{2}$ sucrose, $\frac{1}{2}$ dextrin, lard)	5	11.8	8.7	17	30 +
831 (sucrose, added casein, lard)	5	11.5	8.0	9	17 +

¹ 4% of 1 : 20 liver extract was added to each diet at the expense of the entire ration. The proportions of the carbohydrate and the presence of 10 parts of lard are indicated in the parentheses.

² 3 of the 4 animals in this group failed to survive the 14-week experimental period.

The average incidence and extent of the carious lesions for each of the groups are shown in tables 2 and 3. The results obtained from the initial and repeat experiments were averaged together unless the susceptibility of the control groups

was different. In the latter case the results from each experiment are presented.

The replacement of sucrose by starch resulted in a marked decrease in the severity of tooth damage, the averages for incidence and extent of lesions being 23 and 70+ and 2 and 7+, respectively. The latter index is comparable to that obtained with dextrin or stock rations. Among the carbohydrates tested as the sole carbohydrate in ration 802, starch and dextrin are the only ones that have afforded protection, as opposed to sucrose, glucose, dextri-maltose, fructose, maltose, or lactose. Honey was fed to one group of animals as the source of carbohydrate (by replacement of the sucrose in ration 802) and some protection was noted.

Animals fed a diet of whole liquid milk have been found to be free of dental caries. Therefore, experiments were conducted to obtain further information about the effect of lactose on caries development. Since a high mortality occurs when lactose diets are fed, rations containing less than 50 parts of lactose were prepared for the purpose of contrasting sucrose and lactose. In earlier work (Schweigert et al., '45b) rations containing $\frac{1}{2}$ dextrin and $\frac{1}{2}$ sucrose as the carbohydrate portion gave results comparable to those with the sucrose basal. In the present work a slight protection was observed with $\frac{1}{2}$ dextrin and $\frac{1}{2}$ lactose. However, the results of $\frac{2}{3}$ dextrin — $\frac{1}{3}$ sucrose and $\frac{2}{3}$ dextrin — $\frac{1}{3}$ lactose were similar (table 2). Other experiments were conducted with diets in which 10 parts of lard were added at the expense of the carbohydrate. The ingestion of such diets with sucrose as the carbohydrate has resulted in partial protection and the positive or negative effect of lactose could be studied. The results obtained with rations 826 and 827 indicated that lactose gave somewhat better protection against caries in these lard rations than did sucrose. Unfortunately, however, only one animal survived the experimental period when ration 826 (44.5 parts of lactose) was ingested (table 3). The interrelationship of the kind and level of carbohydrate and fat may be more clearly

demonstrated when other fats are tested in diets similar to these. Such experiments are in progress.

In order to obtain further information about the effects of added protein as well as of other carbohydrates, namely glucose, dextri-maltose, and dextrin, these constituents were added at the expense of the remaining sucrose in the rations containing 10 parts of lard. On the low fat, high carbohydrate diets, a high caries experience occurred with glucose and dextri-maltose as well as with sucrose. In the rations containing 10 parts of lard, glucose and dextri-maltose resulted in caries indices at least as high as those with sucrose (table 3). Similarly, severe lesions were observed when mixtures of sucrose and dextri-maltose or sucrose and dextrin comprised the carbohydrate portion of the diet (rations 830 and 832, respectively). The latter result is somewhat surprising since dextrin has been found to give a low caries index. In earlier work, a low caries occurrence was found with either coarse or fine dextrin rations. Consequently, no attempt was made in the present work to control the particle size of the dextrin used. It is possible that in combination with sucrose the particle size of the dextrin may exert an influence on the initiation of carious lesions in the cotton rat. Ingestion of any of the diets containing lard, however, gave partial protection as compared with the results with animals fed the low fat, sucrose basal ration. Increasing the protein (from 24 to 36 parts) in addition to raising the fat level (ration 831) lowered the caries index from 15 and 32+ to 9 and 17+. Thus, an additive effect of the increased protein and added lard was demonstrated. In earlier work, the effects of the protein and lard were also found to be additive, 50 parts of casein and 10 parts of lard resulting in practically complete protection (1 carious lesion per rat).

These studies confirm earlier observations in that the addition of dextrin, lard, or additional protein at the expense of the sucrose markedly reduced the caries occurrence. Glucose or dextri-maltose as the sole carbohydrate in a low fat diet has been found to be comparable to sucrose in the effect on

dental caries incidence and extent. Similarly, animals fed sucrose, glucose, or dextri-maltose in rations containing 10 parts of lard had a comparable caries occurrence. The effect of lactose appeared to be intermediate between those noted for sucrose and dextrin, although the data are as yet too meager to warrant definite conclusions.

In other experiments the effect of feeding dextrin or sucrose for 14 weeks was compared with that found by feeding dextrin for 4 or 6 weeks and then the sucrose basal ration for 14 weeks. The incidence and extent for the sucrose controls were 27 and 72+ and for the dextrin controls 1 and 2+. However, those that received dextrin rations for 4 or 6 weeks prior to receiving the sucrose ration had a high caries index similar to that of those fed the sucrose ration from weaning. It appears, therefore, that although the animals were older, they were as susceptible to caries as weanlings when fed the sucrose ration for a comparable period. Another series composed of 3 groups was fed the sucrose basal for 14 weeks; the dextrin basal for 10 weeks, then the sucrose basal for 4; and the dextrin basal for 8 weeks, then the sucrose basal for 6. The averages for caries incidence and extent were 23 and 67+; 0; and 10 and 21+, respectively. Feeding the sucrose ration for 4 or 6 weeks was therefore insufficient for maximum caries development.

The average caries index for the offspring from each stock pair fed the sucrose control ration has been computed for approximately 100 animals representing 20 stock pairs during the past year. In general the average susceptibility of the offspring for the entire colony has increased slightly as compared with that of siblings of the parents (Schweigert et al., '45a). The animals obtained from Michigan and Florida have a slightly lower average incidence and extent than the Wisconsin stock. In general the caries susceptibility of the offspring tends to parallel that of the parent stock. No differences in the caries experience of offspring of the stock pairs could be attributed to the number of litters born, the time between litters, or the number of animals in each litter.

SUMMARY

1. The ingestion of diets in which starch or dextrin had been substituted for sucrose resulted in a low incidence and extent of dental caries in the cotton rat as compared with those found after ingestion of the control sucrose ration.

2. Diets in which 10 parts of lard had been added at the expense of the carbohydrate gave some protection as compared with the low fat, sucrose ration. No difference was observed in the effects of glucose, dextrin-maltose, or sucrose in these lard rations.

3. With sucrose rations containing 10 parts of lard, increasing the protein level at the expense of the remaining carbohydrate further reduced the caries occurrence.

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THE UTILIZATION OF CALCIUM IN SOYBEAN PRODUCTS AND OTHER CALCIUM SOURCES¹

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Data have been presented previously (Cahill, Schroeder and Smith, '44) indicating the magnitude of the soybean crop in this country and the portion which could be diverted into channels for human consumption. Although the soybean is grown largely for its oil, of which it contains about 20%, the protein, comprising some 34% of the seed has been shown to be of high quality in human nutrition. In addition, the dry seed is a comparatively rich source of calcium among plant products (0.20%). Inasmuch as this legume promises to become more important in our national dietary, an estimate of the availability of its various nutrients in man is important.

Studies of the utilization by man of the calcium in plant sources indicate that in general, the calcium in most green leaves (lettuce — Mallon, Johnson and Darby, '33; spinach — Bonner, Hunnig, Bates, Horton, Hunseher and Macy, '38; greens — Speirs, '39) compares favorably with that in milk in this respect, whereas the retention from roots (taro — Potgieter, '40; carrots — Breiter, Mills, Rutherford, Armstrong and Outhouse, '42) is somewhat poorer. As regards the availability of the calcium in legume seeds, Pittman ('32) observed that with 5.5 to 6.5 mg of calcium per kg body weight, 80-85% of which was furnished by cooked navy beans, there

¹ Supported by a grant from the Nutrition Foundation, Inc.

was a persistent negative calcium balance in five women subjects. No attempt was made to compare the retention of calcium when it was provided from different sources. In a study of utilization of calcium by three Chinese subjects, Adolph and Chen ('32) fed 8.1 mg per kg body weight of calcium, 80% of which was from cow's milk in control periods and from soybean curd, in experimental periods. On the basis of the calcium balance under these conditions, they concluded that soybean curd and bovine milk are equally effective as sources of calcium in the Chinese diet.

The present report contains data on the efficiency of utilization by adult human subjects of calcium in soybean "milk," in whole cooked soybeans, in evaporated milk, and in calcium sulfate.²

EXPERIMENTAL

The experimental subjects were thirteen male medical students. Data on the ages, body weights, calorie intake, etc., are given in table 2.

Prior to the beginning of the experimental period, the energy requirement was calculated for each subject and proper adjustment then made during the first days of the preliminary period to insure a subjective sense of adequacy of intake. After an initial period of 10 days, the basal experimental period of 20 days followed. The 20-day periods were divided into 5-day groups for analytical purposes and only the last 3 of the 5-day periods were used in making the final calculations in order to avoid the influence of a metabolic lag in the experimental subjects. A total of forty-nine balance periods, each 20 days in length was the source of the data herein presented. Owing to limitation of facilities, there were three separate diet squads of six men each, one squad being studied at a time. There was no indication that the

² Shortly after this study was begun, the Civilian Food Requirements Board of the Food Distribution Administration requested data regarding the availability of the calcium from calcium sulfate. This topic was thus made part of the present investigation.

dietary regime affected the health of the subjects adversely and the changes in body weight were insignificant.

The philosophic approach developed by Steggerda and Mitchell ('39) was used in the evaluation of the efficiency of utilization of calcium in the present study. A negative balance was induced by a calcium-poor ration, in the present case by the preliminary 10-day period plus the first basal 20-day period. The basal period was followed, in most cases, by two 20-day periods in which the basal diet was supplemented with calcium, in the form of a pure salt or as a food. The amount of the calcium supplement was estimated by subtracting the calcium consumed in the basal diet from the total requirement (Steggerda and Mitchell, '41) calculated to the body weight of the subject (9.55 mg per kg body weight). The extent of utilization of the calcium in the tested supplement is computed by equating the spared calcium with the supplemental calcium. In all cases in which the supplement was a food, adjustments in the basal diet were made so as to keep constant the energy value and the protein content of the preliminary low-calcium diet and of the experimental supplemented ration. An adequate protein level was provided (see Kurerth and Pittman, '39; Pittman and Kurerth, '39). Nitrogen balance studies were run on the subjects of Diet Squad I to insure this adequacy of protein level; all members were found to be in nitrogen balance or very nearly so. Three of the diets eaten by one of the experimental subjects are shown in table 1.

The basal diet included bacon, tomato juice, white bread, coffee, oatmeal, apples, cookies, ground beef, canned corn, potatoes, canned applesauce, oleomargarine, and sucrose. The canned foods were of one brand and amounts sufficient for the entire experiment were purchased at one time. The meat was purchased in large quantities, made into 60 gm patties, wrapped, and frozen. Apples and potatoes were purchased as frequently as limited storage facilities required. Foods were sampled periodically and calcium determinations made on aliquots of pooled samples. The supplements used as main

TABLE 1

Sample diets (J.H., 69.1 kg, Diet Squad I).

FOODSTUFF ¹	BASAL DIET				CaSO ₄ · 2H ₂ O SUPPLEMENTED DIET				EVAPORATED MILK SUPPLEMENTED DIET		
	Food		Protein	Calcium ¹¹	Food		Protein	Calcium ¹¹	Food		Protein
	gm	Cal.	gm	mg	gm	Cal.	gm	mg	gm	Cal.	gm
Bacon ²	90	540	11.0	12.2	90	540	11.0	12.2	90	540	11.0
Bread	50	180	4.8	35.6	50	180	4.8	35.6	50	180	4.8
Oatmeal ³	100	391	14.2	47.4	100	391	14.2	47.4	100	391	14.2
Apple ⁴	300	180	0.9	26.1	300	180	0.9	26.1	300	180	0.9
Cookies ⁵	60	260	6.0	16.5	60	260	6.0	16.5	60	260	6.0
Beef ⁶	120	280	22.4	13.1	120	280	22.4	13.1	58	135	10.5
Corn ⁷	150	142	3.8	8.6	150	142	3.8	8.6	150	142	3.8
Potato ⁸	250	200	5.0	27.6	250	200	5.0	27.6	250	200	5.0
Applesauce	200	160	0.4	7.2	200	160	0.4	7.2	200	160	0.4
Tomato juice	200	44	2.0	19.2	200	44	2.0	19.2	200	44	2.0
Oleomargarine	60	440	0.1	11.3	60	440	0.1	11.3	58	425	0.1
Coffee ⁹	360	.	.	13.6	360	13.6	360
Infusion	55	220	0.0	0.0	55	220	0.0	0.0	38	152	0.0
Calcium supplement	2 capsules	.	.	1.2	2 capsules	1.2	2 capsules
CaSO ₄ · 2H ₂ O ¹⁰	1.79	0.0	0.0	415.0
Distilled water	166	231	11.6
Totals		2987	70.6	239.6		2987	70.6	654.6		2990	70.6
Protein calories											
Total			282.4					282.4			
Per cent			9.43					9.43			
Calcium distribution											
Basal food			100%					36.4%			
Supplement			0%					63.6%			

¹ Distilled water was allowed the subjects ad libitum.² The bacon was fried such that the weight-loss represented one-fourth of the original wet-weight. The fat was taken into account in computing food calories. The fat extract was analyzed and no detectable fat was found.³ Based on dry-weight of the oatmeal. The food was cooked with water in the usual manner.⁴ The apples were cored and quartered before eating.⁵ The cookies, made from a milk-free batter, were obtained from a local wholesale bakery.⁶ The beef patties were fried in a minimum of bacon fat.⁷ Drained, canned corn; heated before serving.⁸ Based on wet-weight of the scrubbed, washed potatoes. They were oven-baked before serving and eaten entirely (peel included).⁹ The coffee was prepared by percolation with distilled water. One level teaspoonful of coffee was used per cup; analyses for calcium were based on this infusion.¹⁰ Calcium supplements were divided into three equal portions daily and consumed at mealtime.¹¹ Average calcium values of various pooled food samples during each diet were used in computing food percentages.

sources of calcium in the experimental periods were evaporated milk,³ soybean "milk,"⁴ whole soybeans⁵ and calcium sulfate.⁶

The calcium sulfate, evaporated milk and soybean "milk" supplements each provided all of the added calcium in the appropriate period and were added three times a day at meal-time. However, in the periods where whole soybeans were fed, only half of the added calcium was secured from this source because of the difficulty in consuming the requisite large amounts of this vegetable; the remaining supplementary calcium in these periods was given as calcium sulfate. A capsule containing 1.5 mg of thiamine chloride, 2.0 mg of riboflavin, 1.0 mg of calcium pantothenate, 10 mg of niacinamide, 0.10 mg of pyridoxine and yeast and liver extract, as well as another containing 5000 units of vitamin A and 500 units of vitamin D, were consumed each day by each subject.⁷

The soybeans used as a calcium supplement were prepared by autoclaving the whole beans with two parts of water at 15 pounds pressure for 1 hour. The soybean "milk" was usually diluted with water and chilled before serving. The preparation of the foods of the basal diet may be seen in the footnotes of table 1.

All analyses for calcium were made by a modification of the method of Shohl and Pedley ('22). Calcium-free charcoval markers were used to separate the feces into 5-day periods; the fresh feces were covered with 95% ethanol and dried on steam cones after which analyses were made on aliquots of the dried, ground material. Both food and fecal analyses

³ Whitehouse brand, not irradiated.

⁴ Mull-soy, provided by the Borden Company. Mull-soy is an emulsified concentrate of water, soybean flour, soybean oil, dextrose, sucrose, calcium phosphate, calcium carbonate, salt and soybean lecithin; homogenized and sterilized. Its soybean proteins are practically the nutritional equivalent of egg proteins (Cahill, Schroeder and Smith, '44).

⁵ Run-of-the-mill field-grown soybeans were used rather than a single variety. The soybeans were obtained from the Glidden Company, through the courtesy of Dr. Percy Julian.

⁶ $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, Merck's Reagent.

⁷ Kindly provided by the Gelatin Products Company.

were carried out on samples previously "flamed" and ashed at 550-600°C. Twenty-four-hour urine specimens were collected daily, and an aliquot preserved with hydrochloric acid and toluene. After a 5-day collection period, the pooled urine samples from one subject were again adjusted to volume and aliquots removed for analysis. Preliminary oxidation with ammonium persulfate and nitric acid were made prior to the calcium determinations on the urine samples. The calcium oxalate precipitates were filtered through 10 mm Pyrex micro filter sticks (medium porosity) to exclude calcium losses through ordinary filtration. The precipitates were washed with 1:50 NH_4OH before dissolving in hot normal sulfuric acid and subsequent titration with potassium permanganate.

DISCUSSION OF RESULTS

The level of intake of calcium in the periods when only the basal diet was consumed varies from 153 to 276 mg daily, depending on the energy requirements of the subject. During these basal periods the subjects showed a negative calcium balance (see table 2). Using the value of 9.55 mg per kg body weight per diem (Steggerda and Mitchell, '41) as the calcium requirement of the adult, the total daily need was found; from this value, the daily basal intake was subtracted, the result being the amount of calcium to be added in the form of the supplement under investigation. This procedure should have brought the subject into calcium balance; actually, owing to a lag in securing final analytical data, the subjects were usually in slight negative balance after the supplementation. The decrease in the amount of endogenous calcium loss brought about by the added calcium in the supplement, divided by the added supplementary calcium is the index of availability or utilization of the added calcium.

In general on the basal diet, close to two-thirds of the calcium was excreted in the feces; when calcium sulfate was added, total excretion of calcium was increased but the partition between urine and feces remained unchanged. On the other hand, when evaporated milk or soybean "milk" was

added, the increment in loss was largely by way of the gut. It thus appears that whatever the significance of fecal calcium, the calcium from calcium sulfate was well absorbed under the present experimental conditions and is excreted in greater amounts by the kidney than is the calcium from the other supplements used.

As seen in table 2, the addition of supplementary calcium in the form of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, evaporated milk or soybean "milk," prevents a considerable amount of endogenous calcium loss. It should be pointed out that the evaporated milk was not irradiated and that the soybean "milk," made from a low-fat soybean flour, had been fortified with calcium phosphate. However, adequate vitamin D was given the subjects as a daily supplement to the basal diet.

A total of eleven subjects were used in the study of the utilization of calcium in hydrated calcium sulfate and the final calculations show that under the present experimental conditions the average per cent utilization was 23.7. The values as may be seen in table 2 ranged from 7.2 to 34.6%. With a comparable number of subjects the calcium utilization with soybean "milk" was 22.6% with a range of from 9.9 to 56.1. These values compare favorably with those obtained in the evaporated milk studies. In the latter instance a limited number of subjects (6) was used and the values obtained ranged from 13.7 to 41.8% with an average utilization of 22.6. Only four of the subjects completed the experiment on whole soybeans; however, from the limited data (average per cent utilization was 10.4 and the range was from 2.2 to 18.1) it would appear that not only is the cooked whole soybean not a good source of dietary calcium, but also, when fed in large quantities, it interferes with the utilization of other dietary calcium. A similar action has been observed by Morgan ('34) in human subjects in connection with regenerated cellulose. Inasmuch as little such interference is noted with the soybean "milk," it seems likely that the difficulty may reside in the relatively large amount of roughage in the whole soybeans.

TABLE 2

Utilization of calcium in soybeans and other calcium sources based on the daily average metabolism of calcium

SQUAD AND DATE	SUBJECT AND AGE YEARS	BODY WEIGHT		CALCIUM SUPPLEMENT TESTED	TEST PERIOD DAYS ¹	AVERAGE DAILY METABOLISM OF CALCIUM					BALANCE
		Be- ginning	Weight change			Total calorie intake	Calcium intake		Calcium excretion		
							Basal diet	Supple- ment	Feces	Urine	
Diet Squad I October 12 to December 11, 1943	R.C. 23	kg 85.4	kg - 0.91	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	3697 273 3697 273 3683 264	mg 276 0 276 445 269 436	mg 302 540 530	mg 158 243 688	mg 158 243 171	mg - 15 - 15 - 65
	W.C. 23	76.4	+ 0.91	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	3462 3462 3474	276 276 269	0 445 436	272 516 638	136 220 140	- 132 - 132 - 71
	F.G. 22	62.1	- 0.23	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	2987 2987 2986	226 226 220	0 367 360	189 372 422	245 302 197	- 2.5 - 31 - 39
	J.H. 23	69.1	+ 0.91	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	2987 2987 2990	240 240 233	0 415 406	265 489 516	156 236 169	- 181 - 79 - 45
	P.T. 26	58.4	- 0.45	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	2792 2792 2791	216 216 210	0 324 319	242 454 510	94 129 95	- 159 - 45 - 76
	S.Z. 24	63.6	- 0.45	None CaSO ₄ · 2H ₂ O Evaporated milk	20 20 20	2792 2792 2789	215 215 209	0 380 372	219 475 507	183 199 113	- 152 - 74 - 53
Diet Squad II April 18 to June 22, 1944	F.C. 21	72.6	+ 0.91	None CaSO ₄ · 2H ₂ O Soy milk	20 20 20	3547 3547 3556	237 237 227	0 440 490	260 545 629	112 159 131	- 153 - 47 - 43
	C.C. 21	59.9	+ 0.23	None CaSO ₄ · 2H ₂ O Soy milk	20 20 20	3088 3088 3085	203 203 189	0 354 394	222 461 410	69 83 48	- 43 - 11 + 123
	R.K. 22	71.3	+ 0.45	None CaSO ₄ · 2H ₂ O Soy milk	20 20 20	3367 3367 3372	233 233 233	0 431 481	233 562 608	203 274 197	- 203 - 172 - 91
	M.K. 20	69.0	- 0.91	None CaSO ₄ · 2H ₂ O Soy milk	20 20 20	3160 3160 3155	199 199 190	0 448 498	231 533 631	46 68 58	- 75 + 45 - 1
	P.T. 26	61.7	0.0	None CaSO ₄ · 2H ₂ O Soy milk	20 ² 20 20	3167 3167 3143	202 202 194	0 369 413	194 443 499	79 151 139	- 71 - 25 - 31
	S.Z. 24	63.5	- 0.23	None Soy milk	20 20	2932 2927	197 189	0 592	204 596	107 109	- 114 + 75
Diet Squad III July 13 to September 22, 1944	R.A. 26	100.9	- 1.82	None Soy milk Soybeans and CaSO ₄ · 2H ₂ O	20 20 20	3775 3775 3770	219 194 182	0 795 715 ³	236 831 904	256 261 251	- 273 - 153 - 253
	R.I. 27	55.4	+ 0.91	None Soy milk Soybeans and CaSO ₄ · 2H ₂ O	20 20 20	2266 2275 2265	171 166 154	0 389 359 ³	206 536 491	98 87 93	- 131 - 63 - 71
	C.K. 24	71.8	0.0	None Soy milk	20 20	2845 2817	179 175	0 560	183 663	146 145	- 150 - 73
	P.T. 27	61.7	- 0.45	None Soy milk Soybeans and CaSO ₄ · 2H ₂ O	20 20 20	2796 2796 2801	182 174 160	0 453 409 ³	160 480 484	100 135 110	- 75 + 12 - 25
	S.Z. 25	63.6	0.0	None Soy milk Soybeans and CaSO ₄ · 2H ₂ O	20 20 20	2664 2665 2671	176 168 153	0 476 423 ³	178 561 562	131 126 117	- 152 - 45 - 103

¹ The last three 5-day periods (total 15 days) were used in computing the daily average calcium metabolism and balance.² Inadvertent mixing of samples made use of all 5-day periods (total 20 days) imperative.³ The calcium supplement was supplied half in the form of CaSO₄ and half as whole soybeans.⁴ Subjects P.T. and S.Z. served on all three diet squads. Complete data should be obtained from only five of the members of Diet Squads II and III. S.Z. replaced one of the original members of Diet Squad II and did not participate in CaSO₄ study with this group. One member of Diet Squad III dropped from the squad after the basal diet and lost weight complete either the Mull-soy or whole-soybean experiment. Only four of the subjects completed the whole soybean experiment.

These experiments again show the great variability exhibited by individual subjects (see Steggerda and Mitchell, '46) and suggest that average per cent utilizations of calcium should not be regarded as absolute values but rather serve to show differences in the availability of calcium in the products studied.

SUMMARY

The utilization of the calcium in three foods and one pure chemical compound was studied in adult human subjects. The supplementary calcium consumed ranged from 319 to 795 mg per day. The average per cent utilization of the calcium in evaporated milk was found to be 29.1; in calcium sulfate, 23.7; in soybean "milk," 22.6; whole cooked soybeans, 10.4.

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VITAMIN B₆ AND ITS RELATION TO β -PYRACIN LACTONE IN THE METABOLISM OF THE CHICK

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INTRODUCTION

Hogan and Parrott ('40) postulated the existence of vitamin B₆ after observing the curative effect of liver extracts in correcting both anemia and poor growth in chicks raised on semi-purified rations. Later, Pfiffner and associates ('43), who isolated this vitamin in crystalline form, presented evidence that it would replace liver extracts when incorporated in the basal diet. These workers retained the term vitamin B₆ for this compound. In supplementing these findings Campbell et al. ('44a, b, '45) were of the opinion that relatively large amounts of this vitamin were specifically needed to prevent macrocytic, hypochromic or hyperchromic anemia, leukopenia, thrombocytopenia, poor growth, and poor feathering in chicks. On the other hand, Briggs, Luckey, Elvehjem and Hart ('43, '44, '45), using crude concentrates of liver, concluded that "normal chicks may be obtained with 8 gamma of vitamin B₆ activity, or less, per 100 gm of ration." In addition, Hill et al. ('44) stated that "the folic acid (vitamin B₉) requirement of the chick for growth and the prevention of anemia appears to be less than 15 gamma per 100 gm of diet."

Another and similar factor, active in the nutrition of the chick, namely the L. casei factor from a fermentation residue, was isolated in pure form by Hutchings and co-workers ('44).

Although its absorption spectrum appears to be very similar to that of vitamin Bc (Bloom et al., '44), Scott, Norris, Heuser, and Bruce ('45) showed that α or β pyracin had to be included with it in a purified chick ration to prevent completely macrocytic, hypochromic anemia. They also found that the β -pyracin lactone was more effective than the α -pyracin lactone in promoting the growth of chicks. The observations were confirmed by Daniel, Scott, Norris and Heuser ('45). These workers concluded that folic acid (vitamin Bc) may be an addition product of pyracin and the *L. casei* factor; or that an enzyme system, requiring pyracin and the *L. casei* factor, is related to the formation of folic acid.

Unfortunately we have not had sufficient quantities of the above mentioned factors in crystalline form to conduct parallel experiments. We desire, however, to present some data which indicate first the essential role of vitamin Bc in the nutrition of the chick and secondly, the ineffectiveness of the β -pyracin lactone in supplementing vitamin Bc.

EXPERIMENTAL

Day-old White Leghorn chicks, hatched from eggs obtained from year-old hens which had been kept in close confinement and raised on dry feeds, were weighed, banded, and segregated into groups of ten each. They were housed in metal brooders having raised screen bottoms. The temperature of the air-conditioned room was maintained at 90°F. for the first week of life and then reduced by 5°F. each succeeding week on test until the temperature reached 75°F. The food and water were given ad libitum. The composition of the diets is given in table 1. In addition, a control group of chicks was given a commercial broiler feed¹ enriched with 3% desiccated whole pork liver and 3% dried brewers yeast.

The chicks were weighed periodically, and examined critically for perosis and feather development. An arbitrary scoring system was devised to help evaluate these conditions. If no evidence of perosis existed, no score was indicated. A

¹ Larrowe Milling Co.

slight puffiness of the leg joint without actual slipping of the tendon was scored 1. The most advanced stage of perosis was marked 4. In the accompanying tables the average perotic values represent the score per leg and not per bird. Likewise, feathering was graded in six steps, the normal condition was designated as 0, the poorest feathering as 6. As the development was bilaterally symmetrical, the average represents each bird rather than each wing.

Vitamin Bc crystals (isolated from liver) were dissolved in aqueous 0.005 N NaOH and stored in the refrigerator at

TABLE 1
Composition of basal diets.

INGREDIENTS	NO 59744	NO. 96355	NO 96365	NO. 96375
Cornstarch — gm	52.3	52.3	51.3	51.3
Purified casein — gm	25.0	25.0	25.0	25.0
Gelatin — gm	10.0	10.0	10.0	10.0
Cellu flour — gm	3.0	3.0	3.0	3.0
Salt mixture, Jones and Foster ('42) — gm	5.0	5.0	5.0	5.0
MnSO ₄ · 4H ₂ O — gm	0.1	0.1	0.1	0.1
Succinylsulfathiazole — gm	1.0	1.0
ADEK mixture ¹ — gm	0.1	0.1	0.1	0.1
Vit. A 7520 USP units				
Vit. D (natural) 725 USP units				
mixed tocopherol 32 mg				
menadione — 100 µg				
L-cystine — gm	0.3	0.3	0.3	0.3
Lard — gm	3.9	3.9	3.9	3.9
Choline chloride — gm	0.2	0.2	0.2	0.2
Thiamine ² — mg	0.4	0.4	0.4	0.4
Riboflavin ² — mg	0.8	0.8	0.8	0.8
Pyridoxine ² — mg	0.6	0.6	0.6	0.6
Nicotinic acid ² — mg	2.0	2.0	2.0	2.0
Inositol ² — mg	0.05	0.05	0.05	0.05
Sodium pantothenate — mg	1.1	1.1	1.1	1.1
Biotin — mg	0.02	0.02	0.02	0.02
PABA ² — mg	15.0	..	15.0	..

¹ Added to the diet for the first week of test. Thereafter, these fat soluble vitamins were fed orally as follows. Per 0.1 ml corn oil, per chick per week: vitamin A, 1600 USP units; vitamin D (natural) 160 USP units; α -tocopherol, 6 mg; and menadione, 0.5 mg.

² These B vitamins are mixed in a small amount of lactose (total weight 0.1 gm per 100 gm ration).

0°C. When the chicks required fresh ration about once a week, new lots were made from this stock solution. The lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxy-methylpyridine, termed β -pyracin lactone by Daniel et al. ('45) also was dissolved in water before mixing in the rations. In one experiment, an aqueous solution of the lactone was given daily by pipette to each chick. This solution was made up once a week and stored in the refrigerator. The yeast extract, no. 38843, sometimes referred to as the reference standard, was an aqueous extract of plasmolyzed brewers yeast which had been dried and powdered. Two previous biological chick assays of this material, using crystalline vitamin Bc as a standard, gave values of 54 and 50 gamma of vitamin Bc per gm, respectively.

Experiment no. 1 (vitamin Bc)

The object of this test was to determine whether or not crystalline vitamin Bc would complete the nutritional requirements of the growing chick when fed in combination with the previously known necessary dietary factors. The results are summarized in table 2. After 38 days of feeding the birds receiving 25 gamma of crystalline vitamin Bc per 100 gm of ration weighed 393 gm and a similar group receiving an equivalent amount of vitamin Bc as a yeast extract weighed 355 gm. This is considered to be a normal growth range for these birds.

Experiment no. 2 (vitamin Bc with β -pyracin lactone)

The second test was designed to determine whether or not β -pyracin lactone would supplement vitamin Bc in the nutrition of the chick. Three groups of chicks receiving 15, 50, or 150 μ g of β -pyracin lactone per 100 gm of diet, respectively, were run as controls. A fourth group received 50 μ g of β -pyracin lactone together with 15 μ g of vitamin Bc per 100 gm of ration. This level of vitamin Bc was known to be suboptimum in its action on growth and hemopoiesis. The results are reported in table 3.

TABLE 2
Influence of vitamin B₆ on chick growth.

LEVEL OF VIT. B ₆ PER 100 GM RATION ¹	NO. OF CHICKS		AVERAGE WEIGHT (GM) DAYS ON RATION				
	♂	♀	0	13	20	31	38
I. Vitamin B ₆ crystals no. 91975							
0 µg	1	4	41	90	107	140	153
5 µg	3	3	40	93	126	151	174
15 µg	3	6	39	94	135	222	243
25 µg	6	0	41	107	180	306	393
II. Yeast extract no. 38843							
5 µg ¹	4	4	42	99	133	183	219
15 µg ¹	5	5	39	94	130	189	252
25 µg ¹	9	1	39	104	154	262	355
200 µg ¹	10	0	38	117	176	322	419

¹ Basal ration no. 59744.

¹ These are equivalent vitamin B₆ levels according to previous chick bioassay results. See text for description of no. 38843.

TABLE 3
The ineffectiveness of β-pyracine lactone on the vitamin B₆ syndrome in chicks.

NATURE OF TEST GROUPS	TREATMENT LEVEL PER 100 GM OF RATION (µg)	NO. CHICKS		AVERAGE BODY WEIGHT		4-WEEK DATA			
		Start- ed	Sur- vived	3 wk.	27 da.	Perosis score per leg	Feather- ing score	Hemato- crit (vol %)	Hemo- globin (gm %)
Basal diet, no. 59744	..	10	8	116	134	3.2	5.3	18.4	5.33
β-Pyracine Lactone	15	10	6	97	110	3.3	6.0	16.5	4.54
β-Pyracine Lactone	50	10	8	104	119	3.2	5.3	18.8	5.87
β-Pyracine Lactone	150	10	8	89	95	3.6	6.0	15.3	4.09
Vitamin B ₆ crystals	15	10	9	133	171	2.9	4.7	28.1	8.02
Vitamin B ₆ crystals + β-Pyracine Lactone	15 50	10	9	125	173	3.1	4.8	25.0	7.36
Reference Standard (Aqueous extract of yeast). no. 38843	5 ¹ 15 ¹ 30 ¹	10	9	118	144	3.6	5.9	22.7	6.40
		10	8	135	157	3.3	4.9	26.5	7.77
		10	8	186	219	3.3	3.3	31.6	8.67

¹ Based on chick assay. See footnote table 2.

Three β -pyracin lactone control groups fed the basal diet plus the levels of 15, 50 or 150 μg per 100 gm of ration showed no increase in growth response or improvement in hemopoiesis when compared with the chicks receiving the basal diet.

Vitamin Bc at 15 μg per 100 gm of ration produced chicks which averaged 171 gm per group after 27 days on test. The addition of the lactone produced no measurable increase in growth nor change in perosis, feathering or blood picture. Therefore, it was concluded that no biological activity could be attributed to β -pyracin lactone under the conditions noted in this experiment.

Experiment no. 3 (vitamin Bc, β -pyracin lactone and succinylsulfathiazole)

Since the foregoing tests indicated that β -pyracin lactone had no supplemental effect on our regular anemia-producing ration, the possibility still existed that under similar conditions succinylsulfathiazole, with or without p-aminobenzoic acid, might have some influence on the vitamin Bc syndrome in chicks. Accordingly, ration no. 59744 was modified to contain 1% of the sulfa compound (table 1). The lactone was given by pipette to each chick at a level of 5 μg per day which was equivalent to 50 to 60 μg per 100 gm of ration based on the average food intake for these animals of 8 to 10 gm per day. Further, in order to demonstrate more clearly the lack of supplemental action of β -pyracin lactone, a lower level of vitamin Bc, namely 10 μg per 100 gm of ration, was employed. By these modifications a dietary regime was produced which was similar to the one used by Scott et al. ('45).

In table 4 the resultant data are given. In confirmation of experiment no. 2, above, β -pyracin lactone showed no anti-anemia or growth activity when fed in conjunction with crystalline vitamin Bc in our regular ration, no. 59744, or in the succinylsulfathiazole basal ration (with or without PABA).

TABLE 4

Growth and hemoglobin-promoting response to crystalline vitamin B₆ in chick diets with and without sulfa drugs.

DESCRIPTION OF TEST GROUP	VITAMIN B ₆ LEVEL/100	NO. CHICKS		3-WEEK DATA		
	GM OF RATION (AGE)	Start- ed	Surviv- ing ¹	Body weight (gm)	Hemato- crit (vol %)	Hemo- globin (gm %)
A. Ration no. 59744 — with PABA, without succinylsulfathiazole						
(a) Basal ration	0	11	7	97	15.3	4.50
(b) β -pyracin lactone ²	0	10	5	79	15.2	4.41
(c) β -pyracin lactone ² } + Vit. B ₆ cryst. }	10	10	8	110	16.1	4.83
(d) Vit. B ₆ cryst.	10	10	7	110	19.0	5.25
(e) Vit. B ₆ cryst.	25	10	10	151	28.7	7.74
(f) Yeast extract ³	10	10	9	136	19.9	5.45
(g) Yeast extract ³	25	10	10	140	25.9	7.37
(h) Yeast extract ³	40	10	10	182	30.0	7.68
B. Ration no. 96355 — without PABA and without succinylsulfathiazole						
(a) Basal ration	0	10	4	80	16.5	4.22
(b) Vit. B ₆ cryst.	10	10	7	111	10.0	4.51
(c) Vit. B ₆ cryst.	25	10	10	153	27.9	7.50
(d) Yeast extract ³	10	10	8	104	18.9	5.56
(e) Yeast extract ³	25	10	8	136	25.8	7.13
C. Ration no. 96965 — with PABA and succinylsulfathiazole						
(a) Basal ration	0	10	6	94	19.0	5.45
(b) Vit. B ₆ cryst.	10	10	9	118	20.7	5.90
(c) Vit. B ₆ cryst.	25	10	8	171	29.5	8.18
(d) Yeast extract ³	10	10	9	122	18.7	4.80
(e) Yeast extract ³	25	10	10	146	23.7	6.63
D. Ration no. 96375 — without PABA, with succinylsulfathiazole						
(a) Basal ration	0	10	4	70	13.0	3.54
(b) β -pyracin ²	0	10	5	77	13.8	3.78
(c) β -pyracin ² + } vit. B ₆ cryst. }	10	10	9	101	20.2	5.28
(d) Vit. B ₆ cryst.	10	10	10	114	18.8	4.92
(e) Vit. B ₆ cryst.	25	10	10	144	20.2	7.18
(f) Yeast extract ³	10	10	9	94	19.1	5.16
(g) Yeast extract ³	25	10	10	131	24.4	6.37
E. Commercial broiler						
Ration, fortified ⁴	105	10	9	155	29.4	7.92

¹ Age of chicks— 3 weeks.

² 5 μ g per chick per day.

³ No. 38843. See footnote table 2.

⁴ 3% whole desiccated pork liver (Wilson's) plus 3% dried brewers yeast (Anheuser-Busch, strain G) added.

DISCUSSION

The foregoing data confirm earlier conclusions from this laboratory, presented by Pfiffner and associates ('43) and Campbell et al. ('44a, b, '45), that vitamin Bc *per se* is an important essential in the nutrition of the chick. These findings are in opposition to those of Briggs et al. ('43, '44, '45) and those of Hill, Norris and Heuser ('44). The discrepancy, however, may be explained by the fact that the microbiological assay used by these laboratories did not determine the total amount of vitamin Bc activity but, for the most part, only the free form of the vitamin present in the supplements fed the chicks. For example, in this laboratory microbiological investigation of the vitamin Bc conjugate indicates that treatment of a yeast extract by autoclaving at pH 4 for 12 hours will release only 10 to 15% of the total vitamin present. (Unpublished data — O.D.B.) Likewise, Bird et al. ('45) have shown that vitamin Bc cannot be completely liberated from its conjugate by the taka-diaxase and papain digestion procedure of Cheldelin et al. ('42). The possible conclusion, then, is that Briggs and associates ('45) in reality fed their chicks more vitamin Bc than was indicated. On this basis one may conjecture that the B₁₁ factor of Briggs et al. ('43) is a vitamin Bc conjugate. Likewise, a similar condition existed with the experiments of Hill et al. ('44). In this work the concentrate fed the chicks contained factors R or S. In each case the factors were found to possess anti-anemic properties. It is probable that there is some vitamin Bc conjugate in these fractions. Moreover, since their data showed that factor S at a level equivalent to 15% yeast was nearly equal in hemato-poietic activity to factor R at a level equivalent to 5% yeast, one may estimate that factor R contains at least three times as much vitamin Bc activity as does factor S.

Furthermore, the absolute amount of vitamin Bc activity required by the chick is open to question. For example, our first experiments suggested that for normal response relatively high levels of the vitamin, 100 to 400 μ g per 100 gm of ration, were necessary, while our second test (Pfiffner

and others, '45) showed that maximum growth could be obtained with but 25 μ g per 100 gm of ration using the free form, or an equivalent amount of vitamin B₆ conjugate. Peak blood values were observed at the 200 μ g level but this experiment did not prove that a smaller quantity, somewhat greater than 25 μ g, would not have been in the optimum range. The work reported in table 2 suggests that 25 μ g more nearly approached the optimum requirements for growth. This value is in line with the results of Briggs et al. ('45), who reported that 50 μ g of the L. casei factor (type not designated) per 100 gm of ration produced normal growth and normal hemoglobin formation.

In the present tests there are two factors which may have had a bearing on the results: First, on investigating the source of the chicks used, it was noted that the chicks in the first experiment were hatched from pullet eggs, while in the present investigation the chicks came from eggs produced by more mature hens of the same strain of White Leghorns. Secondly, it is possible that seasonal variation may have been a contributing factor. The first experiments were completed in the late fall and early winter while those reported at this time were carried out in the late spring. If a true growth requirement is to be established, the above points should be considered. Actually in our routine vitamin B₆ assays of liver and yeast preparations (unpublished data) it has been found that the hematopoietic response is a more reliable index of biological activity than is growth. The variation from season to season and between groups of chicks is not as marked. This fact may indicate that the primary role of vitamin B₆ is concerned with the blood forming organs and that growth is of secondary consideration.

Again, the basal ration with and without succinylsulfathiazole and/or p-aminobenzoic acid caused little or no change in the growth of the negative control groups. This is in direct contrast to the results reported by Briggs et al. ('43). These authors reported that growth and feathering on their basal diet was retarded at least 50% by the feeding of suc-

cinylsulfathiazole. The explanation for this difference may be due to our use of corn starch as a source of carbohydrate rather than dextrin. Also, there should be noted in our experiments the possible effectiveness of p-aminobenzoic acid in promoting growth or hemoglobin formation by its stimulation of the intestinal bacteria to produce other unknown factors (table 4).

Finally, in considering the role of β -pyracin lactone in the nutrition of the chick, it is interesting to compare the points of similarity between the published data of Scott et al. ('45) and some of our own data (3-week, table 3). The growth chicks receiving the basal diet plus β -pyracin lactone ranged from 91 to 100 gm as reported by the Cornell investigators while the growth of our chicks ranged from 89 to 104 gm. Both laboratories used chicks which reached approximately the same maximum weight; the Cornell investigators, using a well balanced grain chick ration, obtained birds averaging 192 gm, while our birds receiving the fortified liver-yeast diet, averaged 186 gm at the end of 3 weeks.

If the analogy between the two investigations is carried farther, it may be noted that in our laboratory where 50 μ g of β -pyracin lactone and 15 μ g of vitamin Bc were used per 100 gm of ration, a growth of 125 gm was obtained by the end of 3 weeks, whereas the Cornell authors, using 25 μ g of the lactone and 25 μ g of the L. casei factor (LCF) reported a body weight of 105 gm. When they doubled the quantity of LCF and β -pyracin lactone (50 μ g of each per 100 gm of ration), they obtained a growth of 144 gm in the 3-week period. This would indicate that 15 μ g of vitamin Bc gives a growth response intermediate between 25 μ g and 50 μ g of the LCF. Since we have already indicated in experiment 2 that β -pyracin lactone had no supplementary effect on vitamin Bc and the Cornell investigators have reported that β -pyracin lactone supplements the LCF, this suggests that the LCF is not identical with vitamin Bc compound isolated from liver. The validity of this reasoning can only be proven when the LCF (fermen-

tation), β -pyracin lactone and vitamin B₆ compounds are compared simultaneously in the same laboratory.

In this connection it is interesting to note that Daft and coworkers ('45) have also discussed the possibility of the difference between a fermentation L. casei factor and the liver L. casei factor; and that Day and others ('44a, b) have proven the conjugate nature of the fermentation product.

CONCLUSIONS

Based upon the experimental conditions as carried out in these laboratories the following conclusions are warranted.

1. Crystalline vitamin B₆ is required by the chick for normal growth, feathering and hemopoiesis. This confirms our previous findings.

2. In the presence of a sub-optimal amount of crystalline vitamin B₆, β -pyracin lactone has no supplemental effect on growth, feathering and hemopoiesis of the chick, even in the presence of succinylsulfathiazole (with or without PABA). This suggests that L. casei factor from a fermentation residue is not the same as vitamin B₆.

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THE NUTRITIVE VALUE OF CANNED FOODS

CHANGES IN THE VITAMIN CONTENT OF FOODS DURING CANNING¹

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The vitamin content of canned foods has been a matter of increasing interest during the past 20 years. The results of earlier investigations showed that many canned foods are relatively good sources of some of the vitamins. The more recent studies of Pressley, Ridder, Smith and Caldwell ('44), Ives, Wagner, Elvehjem and Strong ('44), Thompson, Cunningham and Snell ('44), and Ives, Zepplin, Ames, Strong and Elvehjem ('45) have further indicated the contribution that canned foods make in supplying the human vitamin requirements. This extensive series of studies covered more than thirty different food products and involved over 800 samplings of canned foods of known origin (Clifcorn, '44). In addition to indicating the relative richness of the various canned foods with respect to the several vitamins, the data reported also indicate a rather wide variation in the amounts of these vitamins found in different samplings of the same

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product. Whether such variations were due to differences in the variety of the particular product canned, to differences in soil or climatic conditions under which the products were grown, to differences in the procedure employed in canning, or to a combination of these factors could not be ascertained from the data reported by the above authors. It seems reasonable, however, to suspect that some of the variations in vitamin content were at least partially due to differences in canning methods. It also seems logical to suspect that, if those canning conditions could be ascertained which are conducive to extensive vitamin losses, modification of the methods might be effected which would result in the production of more nutritious canned products.

A number of investigators have reported data which indicate that the vitamin content of canned foods is adversely affected by various conditions encountered previous to and during processing. Among these are: Fellers, Esselen and Fitzgerald ('40); Adams ('42, '44), Ross ('44); Clifcorn and Heberlein ('44); Greenwood, Kraybill, Feaster and Jackson ('44); Moore, Wiederhold, Atkins and McDowell ('44); Wagner, Ives, Strong and Elvehjem ('45); Robinson, Stotz and Kertesz ('45); and Poe and McGuire ('45).

From the contents of the above reports there is ample justification for continuing researches along these lines in an effort to determine some of the factors or conditions associated with food canning procedures which are conducive to high vitamin retention, in order that canning methods may be improved to the point of furnishing more nutritious canned foods. The present studies were conducted with the hope of obtaining additional information regarding these questions. Collaboration and cooperation of various canners in the states of New York, Delaware and Maryland during the canning seasons of 1943 and 1944 made possible a study of the effects of some of the various steps in commercial canning procedures on the vitamin losses from nine vegetables and one fruit. While the investigation involved cooperation with canneries located in the three states, the major portion of the investiga-

tion relating to canning conditions was carried out in western New York.³

EXPERIMENTAL

The actual technical studies at the canneries involved the procuring of representative samples at various points along the canning line, the determination of ascorbic acid and the preparation of appropriate samples for the assay of carotene, thiamine, riboflavin, niacin or for total solids. These latter determinations were carried out in the home laboratory at a later date. The various technical operations at the several plants were greatly facilitated by means of mobile laboratory equipment which could be readily transported from plant to plant. In every instance the canning procedure was the one usually in practice in the plant under consideration. An attempt was made to study canning operations in as many different plants as possible, regardless of plant capacity or peculiarity of canning practices. Canning operations in twenty-seven different plants were investigated. Some plants were examined with respect to a single survey involving one product while other plants were investigated with respect to as many as five different products, involving one or more surveys with each product. Two types of surveys were conducted: (1) an "over-all" survey where the product was sampled as it entered the canning line and again as it emerged as a finished product and (2) a "stage" survey where samples were taken at various points along the canning line, including the finished product.

The products studied were: asparagus (spears and center cuts), green beans (cut and whole), wax beans (cut), lima beans, carrots (diced), whole kernel yellow corn (brine- and vacuum-packed), peas (sweet and Alaska), tomatoes, tomato juice and red sour cherries (pitted). In each instance an

³ The authors are especially indebted to the officers and the staff of the Research Laboratory of Birdseye-Snyder Division, General Foods Corporation, Albion, N. Y., who kindly placed laboratory facilities at the disposal of the authors and who also made many of the contacts and arrangements for cooperative studies at the various canneries in that area.

effort was made to secure data concerning the previous history of the product, with special reference to variety, date of planting, soil type, type of fertilization, growing conditions, harvesting date and yield per acre. The method of blanching, blanching temperature and blanching time were also recorded. In many instances it was possible to follow the product from the time it left the farm until it had passed through the entire canning procedure.

Methods of sampling

A uniform batch of the raw product of sufficient size to yield at least 300 pounds of the canned food constituted each sampling. Preliminary studies were carried out in each plant for the purpose of determining the time required for each product to pass from one sampling point to the succeeding point. Without altering the usual canning procedure, the experimental batch was introduced into the canning line at some time during the day when there was a break in the continuity of operation such as that which usually occurs when it is necessary to change from one variety or grade of product to another or immediately following the lunch hour.

The samples which were taken at the various stations along the canning line consisted of at least 2 kg of the material. To secure the final sample twelve no. 2 or no. 2½ cans or six no. 10 cans of the canned product were used. Half of the cans were opened and sampled immediately after cooling, while the remaining cans were stored at a low temperature until subsequently examined at the home laboratory. Samples for ascorbic acid determinations were stabilized with metaphosphoric acid and assayed immediately, while samples for carotene, thiamine, riboflavin and niacin determinations were stabilized by methods which will be described later. All stabilized samples were stored under refrigeration until the vitamin analyses had been completed.

Representative samples for total solids determinations were prepared by placing equal weights of the product and of distilled water in a no. 2 can, closing the can and sterilizing

by autoclaving. Total solids were subsequently determined in the home laboratory by pureeing the contents of the can in a Waring Blendor and reducing a weighed aliquot to constant weight in a Brabender Moisture Tester which was operated at 85°F.

Methods of vitamin assay

Ascorbic acid was determined immediately after sampling by direct titration with 2, 6-dichlorobenzene-indophenol according to a procedure previously described by Vavich, Stern and Guerrant ('45) for peas, except for the following modifications which were found necessary in the case of fresh asparagus and sour cherries, owing to the presence of interfering pigments. The metaphosphoric extract of fresh unblanched asparagus was found to contain a pinkish coloration which interfered with the usual direct titration procedure. This pigmentation appears to be destroyed by heat so that the determination of the ascorbic acid content of blanched or processed asparagus by direct dye titration is not a serious problem. To determine the ascorbic acid content of fresh asparagus, different but measured amounts of the dye solution were added to each of a series of aliquots of the asparagus extract. Sufficient dilute NaOH solution was then added to each aliquot to bring the pH to about 6.5. At or above this pH an excess of the indophenol dye changes to form a blue coloration. Using the presence or absence of the blue coloration as the criterion, it was possible to determine the proper end-point.

The extraction of a red coloration from sour cherries by the metaphosphoric acid solution also made it impossible to use the direct titration method for determining the ascorbic acid content of this product. This red pigment, however, is not extracted from metaphosphoric acid solution by xylene. It was possible, therefore, to determine the proper titration value by measuring the smallest volume of the indophenol dye solution that would produce a pinkish coloration in the xylene layer when the cherry extract-dye solution was shaken with

xylene. Obviously this pinkish coloration is due to an excess of the dye and, therefore, indicates the end-point.

Samples for thiamine, riboflavin and niacin assays were stabilized in N/10 H_2SO_4 , with chloroform and toluene as added preservatives, while samples for carotene assays were stabilized by blending into a puree in the presence of alkaline ethyl alcohol in accordance with the recommendations reported by Feaster and Alexander ('44). The methods employed in assaying carotene, thiamine, riboflavin and niacin were essentially the same as those described in an earlier publication by Guerrant, Vavich and Fardig ('45). Since all of the products studied were not considered rich sources of all of the vitamins, they were assayed only for those vitamins which they were known to contribute in substantial amounts to the human diet. While the samples for carotene, thiamine, riboflavin and niacin assays were stored under refrigeration pending analysis, every effort was made to complete the vitamin assays at the earliest possible date. With the majority of the samples this was accomplished within 10 days or 2 weeks from the dates the samples were taken.

Data

The data obtained in this series of studies have been condensed so far as general characteristics will permit and are presented in table 1.

DISCUSSION

It was obvious that all of the detailed data could not be included, hence condensations and deletions were essential. Among some of the various factors involved in the study such as variety of product, soil type, growing season, yield per acre, canning plant, manufacturer's grade, can size and station of sampling, the data indicated that variety, can size and station of sampling were the most important insofar as their effects on vitamin retention were concerned. For this reason, these latter factors have been retained in the tabulation (table 1) and the data have been condensed without regard

to soil type, fertilization, growing season, yield per acre, canning plant, or manufacturer's grade. It is hoped that these supplementary data may be published in an appropriate form at a later date.

Since there were frequently moisture additions or losses during the various stages of canning, vitamin values calculated to the "as assayed" basis are not necessarily true measures of vitamin retention or vitamin losses. In consequence, vitamin potencies were also calculated on the basis of "dry weight." However, in this calculation, no correction was made to compensate for the added sugar and salt. Since appreciable amounts of water-soluble substances are frequently lost during canning, especially during blanching in water, vitamin values expressed in terms of dry weight are also lacking in finality so far as they reveal the actual effect of the canning operation on the vitamin content of the finished product. Inasmuch as the vitamin content of canned foods is usually expressed in terms of "as assayed" or on the "dry weight" basis, both values are included in the above tabulation. Supplementary studies are now under way in an attempt to determine whether other constituents of canned foods (alcohol insoluble solids, crude fiber and calcium) constitute a more reliable basis for computing vitamin retention.

Asparagus

Two samplings of asparagus were investigated. The samplings consisted of spears and center cuts, packed by the same canner on the same day and from the same batch of raw stock. Each sampling of asparagus was steam blanched for 1.25 minutes, spray rinsed, sorted and hand packed in cans, the spears being packed in no. 2 cans and the center cuts in no. 10 cans. After adding salt, the cans were steam exhausted, sealed, processed in a vertical retort at 248°F. for 15 minutes for the no. 2 cans and for 35 minutes for the no. 10 cans and then cooled.

It may be noted from the data presented in table 1 that the center-cut asparagus originally contained about one-half

TABLE 1

Changes in the vitamin content of canned foods during preparation and processing.

PRODUCT	VARIETY	CAN SIZE	STATION OF SAMPLING	NUMBER OF SAMPLES	VITAMIN CONTENT (MG/100 GM)									
					Ascorbic acid		Carotene		Thiamine		Riboflavin		Niacin	
					As assayed	Dry wt., %	As assayed	Dry wt., %	As assayed	Dry wt., %	As assayed	Dry wt., %	As assayed	Dry wt., %
Asparagus (Spears)	Unknown	2	Before blancher After canning	1 1	50.6 22.3	538 297	0.62 0.51	6.5 6.8	0.23 0.12	2.5 1.6	0.16 0.14	1.7 1.8	1.74 1.50	19.3 20.1
Asparagus (Center cuts)	Unknown	10	Before blancher After canning	1 1	26.0 11.5	325 164	0.26 0.19	3.2 2.7	0.14 0.08	1.8 1.0	0.08 0.06	1.0 0.8	1.02 0.88	13.4 13.2
Beans (Whole green)	Refugee	2	After snippers After canning	7 7	10.1 2.5	101 44	0.21 0.13	2.4 2.2	0.11 0.04	1.3 0.7
Beans (Whole green)	Tendergreen	2	After snippers After canning	1 1	6.8 1.7	81 35	0.46 0.26	5.5 5.3	0.07 0.03	0.9 0.5
Beans (Cut green)	Refugee	2	After snippers After canning	3 3	11.9 3.8	115 62	0.23 0.11	2.3 2.0	0.10 0.04	1.0 0.6
Beans (Cut green)	Refugee	10	After snippers After canning	3 3	14.6 4.5	141 68	0.20 0.12	2.1 1.7	0.11 0.05	1.1 0.7
Beans (Cut green)	Bountiful	2	After snippers After canning	1 1	18.6 4.8	221 91	0.39 0.20	4.6 3.9	0.11 0.03	1.3 0.6
Beans (Cut green)	Tendergreen	2	After snippers After canning	1 1	6.3 2.3	75 45	0.29 0.16	3.5 3.0	0.11 0.04	1.4 0.7
Beans (Cut green)	Tendergreen	10	After snippers After canning	2 2	13.3 3.9	134 67	0.32 0.16	3.2 2.7	0.10 0.04	1.1 0.7
Beans (Cut green)	Refugee	2	After snippers After canning After blancher After blancher After blancher	1 1 1 1 1	11.2 10.9 7.4 7.4 3.4	113 127 92 91 56	0.21 0.14 0.14 0.16 0.16	2.1 1.8 1.7 1.7 1.7	0.10 0.10 0.09 0.08 0.04	1.0 1.2 1.1 0.9 0.7

Beans (Lima)	Henderson Bush	2	10.1	21	0.09	0.2	0.09	0.2	1.33	2.7
	After viner	1	0.06	0.3	0.04	0.2	0.50	2.2
	After canning	1	3.0	13
Beans (Lima)	Henderson Bush	10	10.1	21	0.30	0.6	0.09	0.2	1.33	2.7
	After viner	1	0.06	0.3	0.04	0.2	0.50	2.2
	After canning	1	3.5	13
Beans (Lima)	Clark's Allgreen	2	28.7	89	0.23	0.7	0.16	0.5	1.53	4.7
	After blancher	2	22.0	71	0.15	0.5	0.15	0.5	1.31	4.2
	After filler	2	9.7	63	0.08	0.5	0.08	0.5	0.70	4.4
	After canning	2	10.1	66	0.05	0.3	0.08	0.5	0.66	4.2
Carrots	Chantenay	10	3.9	36	19.90	187.5	0.07	0.6	0.56	5.3
	After washer	2	3.0	28	18.70	173.4	0.07	0.6	0.50	4.6
	After blancher	2	3.0	28	0.07	0.5	0.48	4.4
	After filler	2	3.2	39	12.80	154.6	0.04	0.5	0.36	4.3
	After canning	2
Cherries	Montmorency	10	8.9	50	0.74	3.9
	From rec. tank	6	5.8	48	0.61	4.7
	After canning	6
Corn	Golden Cross	2	9.4	29	0.22	0.6	0.16	0.5	0.08	0.2	1.83	5.6
	After cutters	4	7.4	25	0.15	0.4	0.08	0.2	1.61	5.4
	After washer	4	5.7	22	0.21	0.7	0.10	0.4	0.06	0.2	1.30	4.8
	After filler	4	5.8	26	0.13	0.6	0.03	0.1	0.05	0.2	1.04	5.0
	After canning	4
Corn (Brine packed)	Golden Cross	10	9.4	27	0.14	0.4	0.08	0.2	1.46	4.3
	After cutters	1	7.0	30	0.02	0.1	0.06	0.2	0.96	4.1
	After canning	1
Corn (Brine packed)	Top Cross	2	9.3	29	0.16	0.5	0.09	0.3	1.54	4.7
	After cutters	1	6.8	28	0.04	0.2	0.07	0.3	0.84	3.4
	After canning	1
Corn (Brine packed)	Tendergold	2	7.8	24	0.16	0.5	0.13	0.4	0.07	0.2	1.62	5.0
	After cutters	1	5.9	27	0.09	0.5	0.03	0.1	0.05	0.2	0.90	4.1
	After canning	1
Corn (Vacuum-packed)	Golden Cross	307	10.3	31	0.30	0.8	0.16	0.5	0.11	0.3	1.84	5.6
	After cutters	2	10.0	30	0.14	0.5	0.10	0.3	1.71	5.5
	After washer	2	9.4	31	0.11	0.4	0.10	0.3	2.01	6.4
	After filler	2	10.1	32	0.20	0.7	0.06	0.2	0.09	0.3	1.63	5.1
	After canning	2

TABLE 1 (Continued)

PRODUCT	VARIETY	CAN SIZE	STATION OF SAMPLING	NUMBER OF SAMPLES	VITAMIN CONTENT (MG/100 GM)									
					Ascorbic acid		Carotene		Thiamine		Riboflavin		Niacin	
					As assayed	Dry wt. ¹	As assayed	Dry wt. ¹	As assayed	Dry wt. ¹	As assayed	Dry wt. ¹	As assayed	Dry wt. ¹
Corn (Vacuum-packed)	Top Cross	307 x 306	After cutters	2	9.1	27	0.20	0.7	0.16	0.5	0.11	0.3	1.84	5.9
			After washer	2	7.2	24	0.15	0.5	0.11	0.3	1.61	5.3
			After filler	2	5.6	18	0.20	0.7	0.11	0.4	0.10	0.3	1.53	4.7
Peas (Sweet)	Surprise	2	After canning	2	6.8	22	0.20	0.7	0.05	0.1	0.10	0.3	1.44	4.7
			After viner	5	26.2	129	0.48	2.3	0.49	2.4	0.12	0.6	2.81	13.6
			After canning	5	9.8	67	0.34	2.3	0.18	1.2	0.11	0.6	1.53	10.4
Peas (Sweet)	Surprise	2	After viner	2	28.6	144	0.50	2.5	0.52	2.6	0.11	0.6	2.63	13.2
			Before blancher	2	28.1	139	0.51	2.5	0.11	0.5	2.61	12.9
			After rinse	2	15.1	83	0.48	2.6	0.36	1.9	0.11	0.6	2.50	13.5
Peas (Sweet)	Laxton	2	After filler	2	15.7	80	0.35	1.8	0.10	0.5	2.31	11.8
			After canning	2	9.4	67	0.34	2.4	0.15	1.0	0.10	0.7	1.95	13.8
			After canning	2	28.4	125	0.59	2.6	0.61	2.7	0.12	0.6	3.36	14.8
Peas (Sweet)	Perfection	2	After canning	2	10.3	76	0.47	3.2	0.21	1.5	0.09	0.7	1.27	9.2
			After viner	1	22.3	96	0.48	2.1	0.59	2.5	0.11	0.5	3.45	14.8
			After canning	1	11.3	70	0.37	2.3	0.22	1.4	0.10	0.6	1.24	7.7
Peas (Alaska)	Super-Alaska	2	After viner	1	33.3	162	0.46	2.4	0.30	1.5	0.13	0.6	2.72	13.2
			After canning	1	10.7	71	0.31	2.1	0.10	0.7	0.06	0.4	0.86	5.7
			After canning	1	33.3	162	0.49	2.4	0.30	1.5	0.13	0.6	2.72	13.2
Peas (Alaska)	Super-Alaska	10	After canning	1	10.7	74	0.30	2.1	0.10	0.7	0.06	0.4	0.87	6.0
			After washer	1	40.5	524	3.94	51.2	0.08	1.1	0.20	2.6	0.42	5.5
			After canning	1	22.5	304	3.90	53.2	0.02	0.3	0.10	1.4	0.40	2.7
Spinach	Heavy Pack	2	After washer	4	40.1	562	3.50	52.0	0.09	1.3	0.22	3.1	0.56	8.0
			After blancher	4	39.0	436	3.50	52.1	0.09	1.0	0.22	2.5	0.40	7.1
			After filler	4	27.3	337	3.56	52.4	0.08	1.0	0.20	2.9	0.47	6.6
Spinach	Heavy Pack	10	After exhaust	4	26.4	303	3.40	52.6	0.06	0.9	0.18	2.3	0.44	6.1
			After canning	4	24.6	348	3.40	53.6	0.05	0.8	0.14	2.3	0.43	6.5
			After canning	4	19.4	572	9.86	19.8	9.37	1.1	0.91	9.7	9.97	4.2

Tomatoes	John Baer	10	After peeler	4	16.5	294	0.55	9.8	0.07	1.2	0.04	0.7	0.60	10.6
		4	After canning	4	14.6	259	0.46	8.2	0.06	1.1	0.04	0.7	0.59	10.4
Tomatoes	Assorted	2½	After peeler	2	16.8	273	0.60	9.7	0.08	1.2	0.05	0.7	0.70	11.4
		2	After canning	2	16.1	272	0.51	8.6	0.07	1.1	0.05	0.7	0.70	11.6
Tomato juice	Assorted	46 oz.	After chopper	1	19.0	352	0.58	10.8	0.06	1.1	0.04	0.8	0.64	11.9
		1	After canning	1	9.9	190	0.42	8.0	0.04	0.9	0.04	0.8	0.62	11.9
Tomato juice	John Baer	10	After chopper	2	18.5	297	0.65	10.4	0.07	1.1	0.05	0.7	0.66	10.9
		2	After canning	2	14.6	239	0.44	6.9	0.06	0.8	0.04	0.6	0.65	10.2
Tomato juice	John Baer	46 oz.	After chopper	2	19.4	308	0.73	11.5	0.08	1.2	0.06	0.9	0.74	11.7
		2	After preheater	2	16.7	257	0.63	9.5	0.08	1.2	0.06	0.8	0.68	10.4
		2	At holding tank	2	15.5	278	0.50	8.9	0.07	1.2	0.05	0.9	0.71	12.8
		2	After filler	2	14.7	256	0.39	7.0	0.07	1.2	0.05	0.9	0.72	12.5
		2	After canning	2	14.7	245	0.41	6.9	0.06	1.0	0.05	0.9	0.69	11.5
Tomato juice	John Baer and Stokesdale	46 oz.	After chopper	2	16.6	270	0.57	9.2	0.08	1.2	0.05	0.8	0.75	12.1
		2	After preheater	2	15.0	237	0.54	8.4	0.07	1.0	0.05	0.8	0.75	11.9
		2	After finisher	2	14.6	217	0.44	8.0	0.03	1.0	0.04	0.8	0.66	11.7
		2	After filler	2	13.2	225	0.44	7.4	0.05	0.9	0.04	0.7	0.73	12.8
		2	After canning	2	13.2	249	0.36	6.8	0.05	1.0	0.04	0.8	0.76	12.4
Tomato juice	John Baer	2 tall	After chopper	2	18.0	282	0.64	9.9	0.07	1.2	0.05	0.8	0.67	10.5
		2	After preheater	2	16.4	262	0.51	8.6	0.07	1.0	0.05	0.8	0.69	10.8
		2	At holding tank	2	13.6	251	0.46	8.5	0.06	1.0	0.04	0.8	0.69	12.8
		2	After filler	2	13.7	262	0.41	7.7	0.06	1.1	0.04	0.8	0.72	13.7
		2	After canning	2	14.0	224	0.38	6.1	0.05	0.9	0.04	0.7	0.65	10.3
Tomato juice	Stokesdale	2	Before chopper	1	20.5	387	0.66	12.4	0.06	1.2	0.05	0.9	0.51	10.2
		1	Before finisher	1	17.7	340	0.58	11.2	0.06	1.1	0.05	0.9	0.63	11.2
		1	After finisher	1	16.1	374	0.51	11.9	0.05	1.1	0.05	1.0	0.60	14.0
		1	After heater	1	13.5	329	0.36	8.9	0.05	1.1	0.04	1.0	0.60	14.6
		1	After canning	1	13.2	269	0.37	7.6	0.05	0.9	0.05	0.9	0.52	10.6
Tomato juice	Stokesdale	10	Before chopper	1	20.5	325	0.56	8.9	0.06	1.0	0.05	0.8	0.56	8.9
		1	Before finisher	1	17.9	271	0.51	7.8	0.06	0.9	0.05	0.8	0.54	8.9
		1	After finisher	1	16.8	306	0.42	7.6	0.05	0.9	0.05	0.8	0.58	10.5
		1	After heater	1	12.6	221	0.43	7.5	0.05	0.9	0.05	0.8	0.53	9.3
		1	After canning	1	12.6	217	0.37	6.4	0.05	0.8	0.04	0.7	0.55	9.5

* Uncorrected for the sugar or salt added during canning.

the concentration of the various vitamins as the spears. Blanching and processing appear to have had the same relative effect on vitamin retention in the two types of asparagus. Ascorbic acid and thiamine retention amounted to approximately 50% of the original vitamin content, whereas carotene, riboflavin and niacin retentions were somewhat higher. Vitamin retention was found to be slightly higher in the contents of the no. 2 cans than in the contents of the larger cans. Whether this difference was due to the nature of the product or to processing time cannot be stated with certainty. Since only slight amounts of soluble solids were lost from asparagus during steam blanching, the two bases of expressing vitamin retention yield data for asparagus which are in good agreement.

Beans (whole green)

Eight samplings of whole green beans, packed in four different canneries, were investigated. These consisted of seven samplings of Refugee beans and one sampling of Tendergreen beans. Seven of the eight samplings graded no. 2 (size) while the eighth sampling graded no. 3. All samplings were water blanched for periods varying from 1.5 to 5 minutes at temperatures ranging from 185 to 190°F., depending on the practice of the canner. The blanched beans were packed in no. 2 cans, processed for 20 to 22 minutes at 240°F. and canals cooled.

The beans constituting all eight samplings sustained considerable loss of vitamins during blanching and processing. Both varieties of beans retained approximately 40% of the ascorbic acid (dry weight basis). The beans of the Tendergreen variety retained higher percentages of carotene and thiamine. The latter differences appear to be due to varietal characteristics rather than to slight variations in the canning method. The Tendergreen variety of beans was initially higher in carotene than the Refugee variety.

Beans (cut green)

A total of thirteen samplings of cut green beans were studied. These consisted of eight samplings of the Refugee variety, four of the Tendergreen variety and one sampling of the Bountiful variety. Eleven of the fourteen samplings were graded in respect to size as no. 4 beans. Eight of the samplings represented beans which were eventually canned in no. 2 cans and five of the samplings were from beans consigned to be canned in no. 10 cans. All beans were water blanched for periods ranging from 1.5 to 6 minutes at temperatures ranging from 195 to 200°F., depending on the practice of the different canners. Final processing of all beans was at 240°F. for 20 minutes for no. 2 cans and 35 minutes for the no. 10 cans. The beans canned in no. 2 cans were canaled while those canned in no. 10 cans were first cooled in the retort and then in the canal.

As with whole green beans, all samplings of cut green beans sustained appreciable loss in vitamin content during canning. Ascorbic acid retention ranged from 41 to 60%, while carotene and thiamine retention ranged from 81 to 88 and 50 to 71%, respectively, when expressed in terms of dry matter. The Bountiful variety, while considerably higher in initial ascorbic acid content, sustained the greatest loss of this vitamin during canning, while the Tendergreen variety was found to be relatively low in ascorbic acid but exhibited good retention of the vitamin. While the initial carotene content (after snippers) ranged from 2.3 mg per 100 gm dry weight for Grade 3 (size) Refugee beans to 1.2 mg per 100 gm dry weight for no. 5 (size) Refugee beans, the percentage of carotene retained after being canned was essentially the same for the two grades of beans. When computed on the basis of dry weight, approximately 70% of the thiamine was retained by the canned beans. Within the limits of these studies, can size, blanching time and blanching temperature did not appear to have a marked effect on vitamin retention.

Beans (cut wax)

Four samplings of cut wax beans were investigated. The beans constituting all four samplings were no. 4 size and were prepared and canned in two different canneries. The beans were water blanched for periods ranging from 2.75 to 4 minutes at 190°F., packed in no. 2 cans, processed for 20 minutes at 240°F. and canal cooled. Vitamin retention expressed in terms of dry weight ranged from 56 to 57% for ascorbic acid, 59 to 62% for carotene and from 57 to 59% for thiamine. While the initial carotene content of the wax beans was less than one-half that of the green beans, the percentage of carotene retained after canning was about the same for both types of beans.

Beans (bush limas)

Four samplings of bush lima beans, representing two varieties, two commercial grades, two can sizes, and the operation of two canning establishments, were investigated. The beans constituting two of the four samplings were "all-green" limas, while the beans constituting the other two samplings were made up of green and white beans, with the white beans predominating. The beans were water blanched 2 to 4 minutes at 190 to 200°F., depending on the practice of the canner. Final processing was at 245°F. for 25 minutes for the no. 2 cans and 40 minutes for the no. 10 cans. The no. 2 cans were canal cooled, while the no. 10 cans underwent preliminary cooling in the retort and subsequent cooling in the canal.

From the standpoint of vitamin retention, 62 to 74% of the ascorbic acid, 26 to 50% of the thiamine, 72 to 105% of the riboflavin, and 78 to 89% of the niacin originally present in the vined beans was retained by the canned product. Not only did the "all-green" lima beans (Clark's Allgreen) contain a greater initial amount of ascorbic acid than the mixed lima beans (Henderson's Bush), they also retained a greater percentage of the vitamin after being canned. However, this difference may have been partially due to differences in the

maturity of the two varieties of beans when canned. The data suggest that thiamine destruction was considerably greater where the beans were canned in no. 10 cans than where no. 2 cans were used. However, the effect of can size was less significant in the case of riboflavin and niacin retention.

Carrots (diced)

Two samplings of Red Core Chantenay carrots were investigated with respect to change in vitamin content during preparation and canning. Both samplings were taken from the same cannery where the final product was being packed in no. 10 cans. The topped carrots were subjected to a 10-minute water blanch at 212°F., cooled, sorted, diced, filled into cans, processed for 25 minutes at 240°F. and canals cooled.

While there was appreciable loss of ascorbic acid from carrots as the result of blanching, there appeared to have been some reducing substance or substances formed during the subsequent heat treatment which interfered with ascorbic acid measurements. When calculated to the dry weight basis, the data suggest a slightly higher ascorbic acid content for the canned carrots than for the washed unblanched carrots. Since ascorbic acid losses were found to have resulted from blanching, the apparent increase in vitamin content must have been due to the formation of substances which interfere with the method of assay. Carotene retention by carrots during canning was quite satisfactory inasmuch as more than 80% of the original carotene content was found in the canned product. Thiamine and niacin retentions were also satisfactory, approximately 74% of the original content of these vitamins being retained by the finished product.

Cherries

Six samplings of Montmorency cherries, as packed in no. 10 cans by four different canners, were investigated with respect to changes in ascorbic acid and carotene content during the canning operation. After being sorted, washed, pitted and drained, the cherries were placed in cans, boiling water was

added, the cans were exhausted for 20 minutes in a water bath at 155°F. and closed and processed for periods ranging from 16 to 23 minutes at 200 to 212°F., depending on the practice of the canner.

Ascorbic acid retention was remarkably high with all samplings of cherries, an average of 96% of the original vitamin (dry weight basis) being present in the canned product. While carotene retention appears to have been even more satisfactory than ascorbic acid retention, there is some question regarding the proper interpretation of the apparent high carotene values. It appears that either carotene was liberated or made extractable from the fruit tissues by the heat treatment or that other pigments were liberated or formed which subsequently interfered with the carotene assay. The writers believe that the latter suggestion offers the more plausible explanation for the apparent high-carotene value of canned cherries.

Corn (brine-packed, whole kernel)

Seven samplings of brine-packed whole kernel corn were investigated. These samplings represented three different varieties of corn, two different can sizes, and involved the canning operations of four different canning establishments. The corn comprising some of the samplings was steam blanched for periods ranging from 2.0 to 2.5 minutes, while the corn constituting other samplings was processed without blanching. While all samplings were subjected to a certain degree of washing during the canning operation, neither the method of washing nor the duration appeared to be standardized among the different canners. After being placed in cans and sealed, the corns were processed at 240 to 250°F. for periods ranging from 40 to 50 minutes for the no. 2 cans, and for 90 minutes for the no. 10 cans. The corn processed in no. 2 cans was canal cooled while the corn processed in no. 10 cans received preliminary cooling in the retort before being canal cooled.

There appeared to be some loss of ascorbic acid during the canning of corn, especially during the early stages of the canning operation. However, during processing at the higher temperatures, it appeared that reducing substances are formed which interfere with the ascorbic acid estimation. This is indicated by the apparent increase in the ascorbic acid content of corn during the final stages of the canning operation, especially where the vitamin content is calculated to the dry weight basis. Loss of carotene from corn during canning was variable and did not appear to bear any consistent relationship to variety, size of can or specific processing method employed. Frequently the carotene content of canned corn (on dry weight basis) was found to be greater than that of fresh corn direct from the cutter. These high carotene values may have been due to the formation of carotene-like pigments during the canning procedure which complicated the carotene assay or they may have been due to the loss of non-carotene-containing solids from the cut corn during the rinsing or washing, preparatory to the final stages of canning, with the result that the concentration of carotene in the residual solids exceeded the destruction of carotene during the canning operation. The latter hypothesis appears most tenable.

Thiamine retention in canned corn was found to be relatively low inasmuch as it ranged from 25 to 34% of that of the fresh corn direct from the cutters. There was some evidence that the steam-blanching corn retained slightly higher percentages of thiamine than did the corresponding unblanching corn. The loss of nonriboflavin-containing solids during the washing of the corn tended to overshadow any decrease in riboflavin content during the canning operation. Niacin retention in canned corn ranged from 72 to 95%.

Corn (vacuum-packed, whole kernel)

The changes in the vitamin content of four samplings of vacuum-packed corn, representing two commercial varieties, were investigated. The two varieties of corn were packed

unblanched in 307 x 306 cans by two separate canning establishments. Golden Cross corn was processed at 250°F. for 40 minutes while Top Cross corn was processed at 240°F. for 50 minutes. Both products were canal cooled. There appeared to be no marked differences in the amount or the percentage of vitamins retained in the vacuum-packed corn and brine-packed corn.

Peas (sweet)

Ten samplings of peas (sweet), representing three commercial varieties and involving the canning procedures of four different commercial canneries, were investigated. The samplings of peas graded either no. 4 or no. 5 in respect to size and were canned in no. 2 tins. All samplings of peas were water blanched for intervals ranging from 3 to 10 minutes at temperatures ranging from 190 to 210°F., depending on the practice of the canner. The blanched and rinsed peas were transferred to cans, brine was added, the cans were sealed and processed at 240 to 242°F. for 35 to 40 minutes and canal cooled.

Ascorbic acid retention ranged from 47 to 73% while carotene, thiamine, riboflavin and niacin retention ranged from 96 to 124, 39 to 57, 110 to 138, and 52 to 104%, respectively, when calculated on the basis of residual dry matter. When the vitamin content was calculated on this basis, peas which were subjected to prolonged blanching (6 to 10 minutes) were found to have sustained the greatest loss in ascorbic acid and in thiamine and what appeared to be the least loss in carotene, riboflavin and niacin. Apparently the latter situation was due to extensive loss in noncarotene, nonriboflavin and nonniacin solids during blanching. This hypothesis is confirmed since higher carotene, higher riboflavin and higher niacin values were obtained in the rinsed peas after blanching than were found in the fresh peas direct from the viner. Blanching appeared to be the major cause of ascorbic acid loss from peas while heat treatment was largely responsible for loss of thiamine.

Peas (Alaska)

Two samplings of peas (Alaska) were investigated. Both samplings were of the same variety (Super-Alaska), were packed by the same canner, but were packed in different size cans. The peas of both samplings (size grades 1 and 2) were blanched for 6 minutes at 190°F., packed in cans, brine was added, the cans were sealed and processed at 240°F., the no. 2 cans being processed for 40 minutes and the no. 10 cans for 50 minutes. The smaller cans were canal cooled while the larger ones were cooled in the retort before being subjected to canal cooling.

With the exception of carotene, vitamin losses from Alaska peas during canning were found to be considerable. Ascorbic acid, thiamine and niacin retention amounted to approximately 45% (on dry weight basis) while riboflavin retention was approximately 70% and carotene retention about 88%. It is possible that vitamin losses from canned peas were somewhat greater than the above figures indicate and that the actual vitamin loss was partially obscured by loss in water-soluble solids during blanching.

Spinach

Five samplings of spinach (Heavy Pack) were investigated. These samplings, which were being packed in two different sizes of cans, represented the products of two different canneries. One packer employed water blanching while the other blanched with steam. With both methods of blanching, the conveyor type of blancher was used. The blanching time ranged from 1.5 minutes with steam to 4 minutes with water at 195°F. Final processing was at 252°F. for 45 minutes for the no. 2 cans and 60 minutes for the no. 10 cans. All cans of spinach were canal cooled.

Carotene retention by spinach, during canning, was quite satisfactory. However, the retention of the other vitamins was less favorable. Ascorbic acid retention ranged from 58 to 62%, while thiamine, riboflavin and niacin retention ranged

from 23 to 25, 54 to 81, and 49 to 78%, respectively, when calculated on the basis of residual dry weight. The ascorbic acid losses appeared to have occurred primarily during the preprocessing stages of the canning operation. In fact there appeared to be a slight increase in the ascorbic acid content of spinach during processing. However, this apparent increase is believed to be due to the formation of reducing substances during the heat treatment which interfered with the method of assay. Thiamine losses could be attributed principally to the final heat treatment. Riboflavin losses could be attributed, primarily, to the blanching operation while niacin losses were found to occur throughout the canning operation.

Tomatoes

Eleven samplings of tomatoes, representing three commercial varieties, three can sizes and the products of six different canneries, were investigated with respect to changes in their vitamin content during canning. Six of the samplings were composed of graded tomatoes (fancy) while the five remaining samplings (packed in no. 10 cans) were not quality graded. The general practice employed by the majority of the packers was to subject the tomatoes to some type of washing, scalding, sorting and trimming before they were packed into cans. In all samplings, salt was added to the can contents before sealing. The cans of tomatoes from one sampling were subjected to a hot-water exhaust for 23 minutes before the cans were sealed. The cans of tomatoes from six samplings were vacuum-sealed, those from three samplings were closed with a steam-jet sealer, while the cans of tomatoes from the three remaining samplings were sealed with the ordinary closing equipment. All cans of tomatoes were processed at 212°F., the small cans being processed for periods ranging from 46 to 60 minutes, while those in the no. 10 cans were processed 110 to 130 minutes. All cans were subsequently canal cooled.

With the exception of the carotene loss from tomatoes in the unsealed cans during prolonged exhausting (23 minutes)

in a hot water bath, vitamin retention in tomatoes during canning was found to be satisfactory. Ascorbic acid retention ranged from 88 to 100% while carotene, thiamine, riboflavin and niacin retention ranged from 84 to 89, 92 to 97, 91 to 100 and 92 to 100%, respectively, when computed on the dry weight basis.

Tomato juice

Eleven samplings of tomato juice were also investigated. These samplings comprise juices from those varieties of tomatoes most commonly employed in tomato juice manufacture. The eleven samplings of juice were processed by six different canners and were packed in four different sizes of cans. The general packing procedures employed by the different canners were somewhat similar. The tomatoes were washed, scalded, trimmed, chopped, preheated, the juice was extracted, transferred to cans, salt was added, cans were sealed, processed and cooled. Four of the six processors preheated the chopped tomatoes to temperatures ranging from 135 to 156°F. and held them at these temperatures for intervals ranging from 15 seconds to 4 minutes before expressing the juices. The expressed juice was then heated to a temperature ranging from 190 to 196°F. and held at this temperature for periods ranging from 30 seconds to 2 minutes before being transferred to the cans. A fifth processor made it a practice to heat the chopped tomatoes to 180°F. and hold them at this temperature for 2 minutes before expressing the juice. The hot expressed juice was transferred directly to the cans. The sixth processor maintained the chopped tomatoes at 190°F. for 35 minutes before expressing the juice. In this instance, also, the hot juice was transferred directly to the cans. Salt was added to the contents of all cans before the cans were sealed. All samplings of juice were processed at 212°F., ten samplings being processed in vertical retorts, while the eleventh sampling was processed on a conveyor belt through a hot-water bath. Processing time varied from 15 to 25 minutes for the smaller cans to 45 minutes for the no. 10 cans.

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DIET OF MOTHER AND HYDROCEPHALUS IN INFANT RATS¹

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ONE FIGURE

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The literature on the relation between the diet of the mother and abnormalities in the offspring has been summarized briefly by Warkany ('44); therefore the earlier papers will not be cited again at this time. Warkany and Schraffenberger ('44a), and Nobaek and Kupperman ('44) have described a congenital malformation in the offspring of female rats which received diets that were deficient in riboflavin. Mellanby ('39) observed abnormal development of the teeth and Warkany and Schraffenberger ('44b) observed malformation of the eyes of newborn rats whose mothers received diets deficient in vitamin A. Warkany ('43) reported that skeletal malformation may be present in 45% of the young borne by rats which received a diet that is deficient in vitamin D. Ross et al. ('44) and Cunha et al. ('44) observed congenital malformations, such as syndactylism, talipes, and paralysis agitans in the offspring of sows which received a deficient ration. Hyde ('40) reported that during one 12-month period approximately 18% of the young in an experimental rabbit colony developed hydrocephalus. The epidemic began and ended unexpectedly. The author was quite certain that the abnormality was not inherited, and was of the opinion that it was not due to the diet.

This report describes the occurrence of hydrocephalus in young rats, as a result of inadequate maternal nutrition.

¹ Contribution from the Department of Agricultural Chemistry of the Missouri Agricultural Experiment Station. Journal Series no. 1008.

EXPERIMENTAL

The experimental animals are albino rats and no individuals have been brought in from the outside since the present colony was established 15 years ago. Brother and sister matings have been avoided in recent years. A considerable number of females were provided with Diet A, which contained no crude vitamin carriers to supply unrecognized vitamins. All of the water-soluble vitamins now recognized, except vitamin B₁₂, were supplied as synthetic compounds. Another group of females received Diet B, prepared by adding to Diet A either 5% of a liver extract fraction or 1% of an eluate of a fuller's earth adsorbate of liver extract. When the females reached maturity they were mated with a male from the stock colony. The composition of Diet A is given in table 1.

TABLE 1
Composition of diet A.

	gm		
Casein, acid-washed		Vitamin A	3000 I.U.
and alcohol-extracted	30	Vitamin D	425 I.U.
Cerelose	52	Alpha-tocopherol	2.5 mg
Wood pulp	3	2-Methyl-1, 4-naphthoquinone	2.5 mg
Salts ¹	5	Thiamine hydrochloride	1.0 mg
Lard	10	Riboflavin	1.0 mg
Choline chloride	0.1	Pyridoxine hydrochloride	1.0 mg
Inositol	0.1	Calcium pantothenate	4.0 mg
P-aminobenzoic acid	0.1	Nicotinic acid	5.0 mg
		Biotin	0.02 mg

¹ The composition of the salt mixture is indicated below:

	gm		gm
CaCO ₃	267	FePO ₄ , 4H ₂ O	20
CaHPO ₄ , 2H ₂ O	416	MnSO ₄ , 4H ₂ O	25
MgCO ₃	25	KI	0.6
MgSO ₄ , 7H ₂ O	30	CuSO ₄ , 5H ₂ O	1.6
NaCl	127	NaF	1.0
KCl	21	Al ₂ (SO ₄) ₃ K ₂ SO ₄ , 24H ₂ O	0.24
KH ₂ PO ₄	315	ZnSO ₄ , 7H ₂ O	0.5
		CoCl ₂ , 6H ₂ O	0.66

In a typical case of hydrocephalus the head is dome shaped and greatly enlarged. The amount of brain tissue is reduced and the brain cavity is filled with serum, which may be either straw-colored or varying shades of pink. Gross examination indicated that the hydrocephalus is internal. In the diagnosis of doubtful cases it is very helpful to observe the transmission of light through the brain cavity. A small box, with an opening 0.25 inches in diameter at one end, was built to enclose a 100 watt light bulb, and the head of the rat is held between the source of light and the observer. A normal brain transmits little or no light, but if a rat has hydrocephalus enough light is transmitted to permit ready detection of advanced cases.

Other symptoms often accompany hydrocephalus, though they do not appear consistently enough to be reliable criteria. The eyes sometime appear to be abnormally small, and in a few cases the eyelids on one side fail to open. Blindness in both eyes has not been observed. If the affected rat survives long enough, muscular incoordination sometimes develops. Hydrocephalus has not been recognized as yet in an animal that was less than 10 days old. It is possible that some of the young on Diet A, which died before they were 10 days old, were affected but death occurred before the symptoms were recognized. Most cases were first observed between the ages of 14 and 20 days, but a few were first discovered at the age of 24 to 28 days. Photographs of typical cases are shown in figure 1, and the observations on the young are summarized in table 2.

A total of 1756 normal young were weaned from the females on Diet A and in addition there were thirty with hydrocephalus. Of these thirty only two survived to the usual weaning age of 28 days. One died 46 days after the condition was first detected. The other was still alive and appeared fairly normal when this manuscript was prepared, 160 days after hydrocephalus was first diagnosed. There were no cases of hydrocephalus in the 1020 young weaned by the females on Diet B, and none in the several thousand young reared by the females in the stock colony. The incidence of hydrocephalus is low

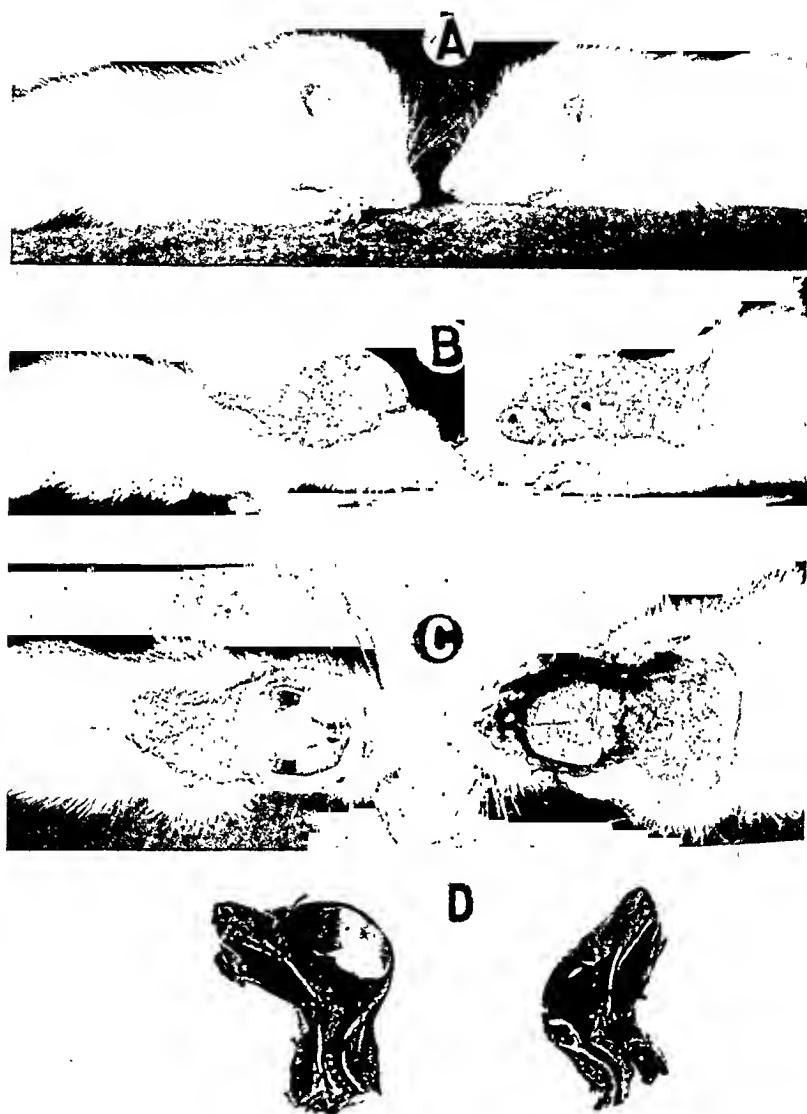


Fig. 1 Hydrocephalic rat on left, normal on the right. A, B, C, photographs of the same rats. D, sagittal section through head of a hydrocephalic and of a normal rat.

but since it has occurred only in young whose mothers received Diet A, it seems reasonable to conclude that the condition is due to a nutritional deficiency. The experimental and control animals were selected at random from the same stock colony and it is assumed that the abnormality has no correlation with genetic constitution. There was one hydrocephalic

TABLE 2

Incidence of hydrocephalus and moisture content of brain of affected animals.

OBSERVATIONS	RATION	
	A	B
Number of females that bore litters	230	54
Number of litters borne	498	175
Number of young weaned	1756	1020
Number	30 ¹	0 ¹
Young with hydrocephalus		
Per cent	1.7	0
Number of litters with 1 case of hydrocephalus	18	
Average number of young per litter	4.7	
Number of litters with 2 cases of hydrocephalus	4	
Average number of young per litter	4.6	
Number of litters with 4 cases of hydrocephalus	1	
Number of young in the litter	5.0	
Observations on the brain		
Number of animals	8 ²	12
Average age, days	19	18
Average weight of rats, gm	20.1	30.1
Average weight of fresh brain, gm	1.68	1.06
Average weight of dry brain, gm	0.13	0.18
Per cent of moisture in the brain	92.1	83.8

¹ Statistical analysis by the chi-square method gave a probability of less than 0.1 that the difference was due to chance.

² All 8 had hydrocephalus.

young in the litter of a female which received Diet A fortified with 5% of dried brewers blended yeast. One could assume that the yeast does not contain a significant amount of the factor that prevents hydrocephalus. There is also no reason to suppose that the yeast supplied any other factor that was deficient in Diet A, for the percentage of young weaned on

Diet A alone was practically as high as when it was fortified with yeast.

The nutrient which prevents hydrocephalus has not been identified. The liver fractions supplied vitamin B₆, and probably unidentified vitamins in addition. Crystalline vitamin B₆ has not been tested by the prophylactic method. Three rats with marked hydrocephalus were each given 500 mg daily of the liver eluate for 4 days, and 2 were each given 100 µg daily of crystalline L. casei factor² for 4 days. All five continued to decline and died. Since the three rats which received the liver eluate did not improve it would appear that hydrocephalus can not be cured after it has once developed.

SUMMARY

Female rats received a synthetic diet to which thirteen vitamins had been added. The vitamin mixture did not contain ascorbic acid or vitamin B₆, and the diet contained no unrecognized vitamins unless they were present as contaminants. Nearly 2% of the offspring developed hydrocephalus. Presumably this abnormality was the result of a nutritional deficiency.

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² Lederle.

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NUTRITIONAL STUDIES WITH THE DUCK

III. NIACIN DEFICIENCY¹

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ONE FIGURE

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In previous reports the performance of young ducklings fed simplified diets (Hegsted and Stare, '45) and a diet deficient in pyridoxine (Hegsted and Rao, '45) has been described. The rapid growth of the duckling compared to its relatively small initial weight apparently makes storage of nutrients a minor factor in the body economy. Those deficiencies which have been studied to date develop with surprising rapidity and often are apparent within 3 or 4 days after the animals are fed the deficient diet.

In this paper are reported the results of studies made with niacin deficient diets.

EXPERIMENTAL

The ration used had the following percentage composition: sucrose 49.7, SMA casein 18, gelatin 10, corn oil 10, salt mixture 5, mono-calcium phosphate 1, cod liver oil 2, liver fraction "L"² 4 and choline 0.3. Crystalline vitamins³ with the exception of nicotinic acid were added at the levels used in

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²Supplied by the Wilson Laboratories, Chicago, Ill

³Supplied by Merek and Co., Rahway, N. J.

previous work (Hegsted and Rao, '45) and alpha tocopherol, 50 mg per kilo, and menadione, 1 mg per kilo, were mixed with the ration. The ration was essentially the same as previously described, although the corn oil content had been increased by 6%.

Day-old ducklings were purchased and fed a commercial mash for 3 or 4 days before they were given the experimental diet. Their average weight when the experiments were started was from 60 to 70 gm.

RESULTS

The average weight of ducklings which received the ration containing increasing amounts of nicotinic acid is shown in table 1. It is seen that great differences in growth were apparent even on the fifth day of the experiment. The total gain of the birds on the purified basal diet was only 40 gm in 20

TABLE 1

The growth response to nicotinic acid of ducklings fed various diets.

DIET	NO. OF DUCKS	NIC. ACID ADDED	NIC. ACID CONTENT (CALCULATED)	AVERAGE WEIGHT			
				5 days	10 days	15 days	20 days
		mg/ 100 gm	mg/ 100 gm	gm	gm	gm	gm
Purified	10	0	.6	83	87	94	100
Purified	4	0.5	1.1	88	117	146	176
Purified	14	1.0	1.6	114	163	219	318
Purified	7	2.0	2.6	145	271	406	530
Purified	7	3.0	3.6	130	280	399	550
Goldberger	4	0	1.0 ²	86	114	130	145
Goldberger	4	5.0	6.0	115	175	282	440
Goldberger + 4% "L" ¹	4	0	1.6	89	124	192	260
Goldberger + 4% "L" ¹	4	5.0	6.6	117	194	297	465

¹ Wilson liver extract fraction "L".

² Calculated from the figure of 1.43 mg of nicotinic acid per 100 gm of yellow corn meal (Committee on Food Composition of the Food and Nutrition Board, Tables of Food Composition, Washington, 1943).



Fig. 1 Ducklings 3 weeks of age fed the simplified diet. The bird on the left received the diet with no added nicotinic acid.

days, compared to approximately 500 gm for those receiving 2 or 3 mg of nicotinic acid per 100 gm of ration.

The minimum requirement appears to be approximately 2 mg of nicotinic acid plus that contained in the ration. According to the figures published by Cannon et al. ('45) the casein should supply only 0.00648 mg per 100 gm of ration while the liver extract supplied 0.64 mg.⁴ The requirement is thus about 2.5 mg per 100 gm of ration.

⁴The figure for the nicotinic acid content of the liver extract was kindly supplied by S. W. Heir, Wilson Laboratories, Chicago, Ill.

The relatively high nicotinic acid requirement is also easily demonstrated by the use of a modified Goldberger ration. This ration contained 71% yellow corn, 18% casein, 4% salts, 5% cottonseed oil, and 2% cod liver oil. Vitamins were added at the same levels used in the simplified ration. The growth on this ration with and without nicotinic acid and liver extract is also shown in table 1. After supplementation with all of the known crystalline vitamins, a diet composed chiefly of corn is severely deficient in nicotinic acid for the duck.

The symptoms of deficiency on both the purified and the Goldberger rations are not striking. The ducks are small, weak (fig. 1), and have diarrhea. The cloaca usually appears large and full of fluid. The eyelids are often stuck together, which may be due in part to the stickiness of the high sugar rations since the condition appears to be more severe on the purified diet. In some of the birds there appeared to be an accumulation of food underneath the tongue and some necrotic tissue underneath this. However, nothing comparable to the black tongue (Briggs et al., '42) or the dermatitis (Briggs et al., '43) which have been described in nicotinic acid deficient chicks was seen.

DISCUSSION

The performance of ducklings fed the Goldberger ration is to be contrasted with that of chicks since Waisman and Elvehjem ('40) were unable to demonstrate a clear-cut response to nicotinic acid in chicks fed this diet and various combinations of the other vitamins. The dog, on the other hand, appears to be more comparable to the duck. For both of these species the Goldberger diet is primarily lacking in nicotinic acid. Whether these differences in response are referable to the low tryptophane content of the corn diet and species differences in the ability to substitute tryptophane for nicotinic acid remains to be investigated. The dog and the chick both develop an inflammation of the tongue and mouth parts while this has not been noted in deficient ducklings. It may be recalled, however, that Handler and Dann

('42) found that under certain conditions black tongue was often not a symptom of dogs dying of nicotinic acid deficiency. Thus the mouth symptoms may be related to factors not obvious at the present time.

Recently Briggs ('46) has reported that the symptoms of nicotinic acid deficiency in the turkey poult are comparable to those found in chicks. Perosis is also reported as a symptom in these two species but was not seen consistently enough in ducklings to be considered the result of nicotinic acid deficiency.

The development of a nicotinic acid deficiency on diets consisting primarily of corn may suggest a consideration of this vitamin in the practical feeding of ducks. It may also be noted that this diet is low in factors supplied by the liver extract, presumably the *L. casei* factor (Hegsted and Stare, '45).

SUMMARY

Young ducklings rapidly develop a nicotinic acid deficiency on purified diets low in nicotinic acid as well as on the modified Goldberger diet containing large amounts of corn. The symptoms observed were lack of growth, diarrhea, and general weakness.

The requirement for rapid growth on the purified diet is approximately 2.5 mg per 100 gm of ration.

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NUTRITIONAL STUDIES WITH THE DUCK

IV. THE EFFECT OF VITAMIN DEFICIENCIES ON THE COURSE OF *P. LOPHURAE* INFECTION IN THE DUCK AND THE CHICK ¹

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Only few experimental data are available on the influence of specific nutritional deficiencies upon the course of malaria. Passmore and Sommerville ('40) placed monkeys on a diet similar to that of the rice-eating poor of India. This diet is suboptimum in at least vitamins A and C and in calcium. The course of malaria (*P. cynomolgi* and *P. knowlesi*) in the deficient animals did not appear to be different from that in the controls. Trager ('43) produced biotin deficiency in ducks and chicks by feeding them a diet containing a large proportion of dried egg white and infected them with *P. lophurae*. For both species of hosts, the average peak parasite number was 50% to 100% higher in the deficient animals than in the controls; the highest peak was always reached in a deficient animal and the lowest in a control; and more of the deficient animals died of the infection. Uninfected controls very rarely died of biotin deficiency. It was shown that the increased parasitemia was not a general effect of malnutrition since pantothenic acid deficient birds were not more susceptible than corresponding controls.

Seeler, Ott and Gundel ('44) confirmed the finding of Trager with regard to biotin deficiency and have extended the studies

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to several other deficiency diseases. Riboflavin deficiency (Seeler and Ott, '44) causes the infection produced by *P. lophurae* to be less severe as judged by the degree of parasitemia. On the other hand, protein deficiency (Seeler and Ott, '45a) and "folic acid" deficiency (Seeler and Ott, '45b) each caused a more severe infection with considerably higher degrees of parasitemia.

In the present report the effects of deficiencies of nicotinic acid, thiamine, choline, and vitamin A upon the course of *P. lophurae* infections in ducks and chicks are presented. As is well known, the normal chick is fairly resistant to this infection. Death seldom occurs unless the inoculum is extremely large. The duckling, however, is very susceptible, and in our experience only an occasional bird survives the disease. We have thus compared the response to the same parasite of a resistant and a susceptible species fed identical diets. As shown previously (Hegsted and Stare, '45), the nutritional requirements of chicks and ducks appear qualitatively similar.

METHODS

Pekin ducklings and white Leghorn chicks were used. The animals were 1 to 7 days old when received from the hatchery. They were housed in wire mesh cages and in most of the experiments given free access to duck pellets and chick mash, respectively, until they had reached a weight of 90 to 130 gm. In a few studies the birds were given the experimental diet when 2 or 3 days old. The animals were then divided into groups of three to nine of comparable weight and put on the experimental diet ad libitum. A simplified ration developed in this laboratory (Hegsted and Stare, '45) was used for both ducks and chicks. The various deficiencies were produced by simply omitting the appropriate vitamin from the diet. Usually two groups of birds received the diet without the vitamin and two groups the complete diet shown in table 1. In some experiments, two additional groups of animals were fed diets containing intermediate levels of the vitamin.

Before infection with malaria, the animals were weighed daily; thereafter, every other day. When the deficiency had become evident in the group on the lowest vitamin level, one group at each vitamin level was infected with malaria, its companion serving as a noninfected control. A decrease in the rate of growth is usually the first evidence of deficiency,

TABLE 1
Composition of the purified diet.¹

	PER KILO
Sucrose	492 gm
Cascin, SMA, "vitamin-free"	180 gm
Gelatin	100 gm
Salts IV ²	50 gm
CaHPO ₄	10 gm
Liver extract ³	40 gm
Corn oil	100 gm
Cod liver oil ⁴	20 gm
Choline chloride	3 gm
Biotin, 100 mg per ml in 50% alcohol	2 ml
Mixture of water-soluble vitamins ⁵	10 ml
Mixture of fat-soluble vitamins ⁶	5 ml

¹The crystalline vitamins were supplied by Merek and Co, Inc., Rahway, N. J.

²Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart, J. Biol. Chem., vol. 138, p. 459, 1941.

³Wilson's Liver Fraction "L"—supplied by the Wilson Laboratories, Chicago, Ill.

⁴For the experiments on vitamin A deficiency, no cod liver oil was added; 120 gm of corn oil and 10,000 I.U. of viosterol were used per kilo of ration. As a source of vitamin A, a concentrated fish liver oil containing 100,000 I.U. per gm was used. This oil contained very little vitamin D.

⁵Containing thiamine hydrochloride, 100 mg; calcium pantothenate, 400 mg; riboflavin, 200 mg; nicotinic acid, 1000 mg; pyridoxine hydrochloride, 100 mg in 250 ml of 95% alcohol.

⁶Containing alpha tocopherol, 1000 mg; menadione, 10 mg in 100 ml corn oil.

and the birds were inoculated 1 to 3 days after this was evident. In studies on vitamin A and thiamine deficiency, it was necessary to supply small amounts of these vitamins in the diet to prevent the animals from dying before the studies were complete. All deficiencies occurred much earlier in the duck than in the chick. This can probably be ascribed to the much more rapid growth of the former animal.

The size of the inoculum in the experiments on ducks varied from 2×10^5 to 10^8 parasites per kilo of body weight, the same amount being used on all animals in the same experiment. Chicks were inoculated with 5×10^8 parasites per kilo of body weight. The parasitized blood was obtained from donor ducks. It was diluted with 0.8% sodium chloride, so that the dose to be administered was contained in 0.2 to 1.0 ml and immediately injected intravenously into the animals. Daily smears were taken, starting 2 or 3 days after inoculation. They were stained with Wright's stain and 500 red blood cells were counted to permit the determination of the percentage of parasitized cells.

RESULTS

Nicotinic acid

Deficiency of this vitamin in chicks manifested itself after 7 days in a slight retardation of growth. The mortality due to the deficiency alone was very low, probably because this diet contains approximately 0.6 mg nicotinic acid per 100 gm (Hegsted, '46). The mortality in both deficient and non-deficient chicks infected with malaria was also low.

The absence of nicotinic acid in the diet has a pronounced effect upon the course of malaria. In the first experiment (table 2), the peak of the parasitemia in deficient chicks was four times as high as that in the controls (42.5% as compared with 11.1%) and occurred 2 days earlier. In the animals that received one-sixth of the full amount of nicotinic acid, the peak was 18.1% and coincided in time with that in the non-deficient group. Almost all animals were able to get rid of their infection by the thirteenth day after inoculation. It is of interest that the deficient group, in spite of the much more severe infection, became free of parasites in approximately the same number of days after infection as the controls. Re-infection with 16×10^8 parasites per kilo did not produce a parasitemia.

A second experiment in which the degree of infection was somewhat less in both groups confirmed the increased sus-

TABLE 2

Average weight and percentage of parasitized red cells of nicotinic acid deficient chicks as compared with nondeficient animals.¹

DAYS	NO NICOTINIC ACID			NICOTINIC ACID 0.5 Mg %		NICOTINIC ACID 3.0 Mg %		
	Group 1		Group 2	Group 3		Group 4	Group 5	
	Weight	Weight	% Parasites	Weight	% Parasites	Weight	Weight	% Parasites
0	114(8) ²	105(9)	.	95(9)		104(9)	118(7)	
7	160	137	Inocu- lated	151	Inocu- lated	162	175	Inocu- lated
10		150	9.7	163	0.9	175	191	0.2
11	172		12.1		1.8			0.6
12		155	39.6	177	4.7	182	202	1.1
13	181		42.5		13.1			3.8
14		152	36.0	185	14.2	195	211	5.9
15			28.7		18.1			11.1
16		157	16.2	185	14.1		223	10.4
17	200	160(8)	9.0	197(8)	8.2	219	230(0)	2.8
19	217	165	1.8	212	3.3	242	246	0.0
21	229	167	0.0	223	6.8	260	254	0.0
23	244	176	0.1	246	0.8	272	287	0.0
			(Rein- oculated)		(Rein- oculated)			(Rein- oculated)
28		187		270			357	
29			0.0		0.0			0.0
33			0.0		0.0			0.0

¹ Groups 2, 3, and 5 were inoculated with 5×10^8 parasites per kilo; groups 1 and 4 served as controls. The animals were 18 days old at the beginning of the experiment.

² Figures in parenthesis = number of animals.

ceptibility of nicotinic acid deficient chicks (table 3). The parasitemia of the controls reached a peak of 3.2% on the sixth day, of the deficient animals 16.6% on the seventh day. Both groups became free of parasites on the eleventh day after infection.

In similar experiments using groups of six to eight ducklings, a consistent difference was not found between the parasitemia of the deficient animals, as compared with that of the controls. Typical results are indicated in table 4. In two

other comparable experiments, the deficient birds appeared slightly more susceptible in one but somewhat less susceptible in the second.

TABLE 3

Average weight and percentage of parasitized red cells of nicotinic acid deficient chicks as compared with nondeficient animals.¹

DAYS	NO NICOTINIC ACID			3 MG PER CENT NICOTINIC ACID		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	104(5) ²	103(8)		104(5)	104(7)	
3	128	119		122	124	
7	143	135		144	144	
8	146	142	Inoculated	161	162	Inoculated
10	152	147	0.1	181	178	0.1
12	179(4)	152(7)	2.9	201	186	1.6
14	180	143	2.2	222	194	1.5
15			2.9			1.8
16			11.1			3.2
17	191	169(4)	16.6	250	212	2.3
18			13.0			1.0
19	206	166	5.7	292	221	0.2
21	220	185	0.0	311	221	0.4

¹ Groups 2 and 4 were inoculated with 5×10^3 parasites per kilo; groups 1 and 3 served as controls. The animals were 19 days old at the beginning of the experiment.

² Figures in parenthesis = number of animals.

Thiamine

The absence of thiamine in the diet of chicks did not appear to influence the course of malaria to a significant degree. The peak of the parasitemia occurred in the twelfth day after inoculation and was 21.7% for the deficient birds, 19.2% for birds receiving the diet plus 80 µg of thiamine per 100 gm of diet, and 21.4% for birds on the complete diet. Severe deficiency symptoms occurred in the first group and 75% of the birds died on the thirteenth day. Growth was suboptimum in the partially deficient group.

Ducks given a ration containing 80 μ g of thiamine per 100 gm began to show weakness of the legs and difficulty in walking after 6 days. This amount of the vitamin was apparently sufficient to prevent convulsive seizures. The animals continued to gain weight, though at a decreased rate as compared with the nondeficient controls. A thiamine deficiency of this extent did not significantly modify the course of malaria. The

TABLE 4

Average weight and percentage of parasitized red cells of nicotinic acid deficient ducks as compared with nondeficient controls.¹

DAYS	NO NICOTINIC ACID			3 MG PER CENT NICOTINIC ACID		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	105(4) ²	108(8)		113(2)	108(6)	
2	118	117	Inoculated	150	153	Inoculated
4	122	132	0.3	220	210	6.3
5			2.3			1.8
6	124	137	4.9	292	266	2.8
7	135	138	18.7	340	275	10.2
8			39.2			31.9
9	131	140	41.6	401	288	42.2
10	125	135(7)	31.4	460	286	31.0
11			15.3			12.6
12	132	141(6)	11.6	552	322(3)	0.5

¹ Groups 2 and 4 were inoculated with 10⁶ parasites per kilo; groups 1 and 3 served as controls. The animals were 8 days old at the beginning of the experiment.

² Figures in parenthesis = number of animals.

peak of the number of parasitized red cells in the deficient group amounted to 41.6% and was reached 11 days after inoculation. In the control group a peak of 50.8% was reached on the same day. All but one of the eight nondeficient ducks died of the infection within the period of observation (13 days), whereas four out of eight deficient animals were alive at the end of the experiment.

Choline

Inconsistent results have been obtained with choline deficient chicks. In two experiments some of the deficient chicks appeared highly susceptible and the deficient groups as a whole were found to have a significantly higher parasitemia than the controls. In two later experiments the same wide variation was found but statistical treatment failed to indicate any difference in susceptibility between the two groups. Differences in the degree of deficiency may account for these differences.

Ducks placed on a ration without added choline showed a retardation of growth after 3 days. After another 5 days, the first deficiency manifestations appeared in the form of weakness of the legs. These symptoms rapidly progressed to typical perosis so that eventually the animals were unable to stand or walk, though they continued to gain weight at a slow rate. Within the 18 days of observation only one out of five ducks died of the deficiency. The course of malaria appeared somewhat milder in the deficient animals; a statistically significant difference was found to exist between the two groups of eight ducks 6 days after inoculation (15.9% parasitemia in the deficient ducks, 32.6% parasitemia in the controls), but the difference was less marked in the succeeding days.

Vitamin A

Chicks on the purified diet without vitamin A showed a normal rate of growth during the first 14 days, as compared with the controls (table 5). A moderate retardation of growth became evident after this period. Nine days later the animals began to lose weight and most of them died in the course of the next 4 days. After 21 days on the deficient diet, the chicks began to show weakness of the legs and ataxia. These symptoms rapidly progressed, so that finally the animals were unable to stand or reach their food. Convulsions were not seen.

Only a few of the nondeficient chicks died of malaria within the period of observation (8 days). The infection ran a milder

course in the deficient animals; the parasite count was consistently lower than in the controls as shown in table 5.

In the ducks placed on a low vitamin A diet, symptoms of the deficiency appeared after 8 days and almost all animals died within 4 days after the first appearance of symptoms. In the

TABLE 5

Average weight and percentage of parasitized red cells of vitamin A deficient chicks as compared with nandeficient animals.¹

DAYS	NO VITAMIN A			10,000 I U VITAMIN A PER KILO		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	71(6) ²	70(8)		65(5)	66(7)	
5	99	99		93	94	
10	130	123		127	125	
14	154	157		167	155	
16	164	169		189	170	
18	176	183		215	193	
19	182	184		224	200	
20			Inoculated			Inoculated
21	186 ³	190 ³		254	209(6)	
23	180	167	1.5	267	215	2.2
24			1.7			4.8
25	161	156(7)	3.3		224	14.6
26	(5)	(5)	10.1			33.8
27	154	169(2)	36.2	318	229	49.9
28	(2)	-	54.0		(5)	51.2
30	(0)	(0)				

¹ Groups 2 and 4 were inoculated with 5×10^4 parasites per kilo; groups 1 and 3 served as controls. The animals were 10 days old at the beginning of the experiment.

² Figures in parenthesis = number of animals.

³ Indicates first evidence of weakness and ataxia of legs which gradually progressed.

studies on malaria, 80 I.U. of vitamin A were added per kilo of ration to keep the birds alive.

No differences were found in the course of malaria of the deficient animals as compared with the controls. In the former, the peak of parasitemia was reached on the seventh day

(76.6%). All ducks had died on the next day. In the controls, the peak (76.1%) occurred on the same day.

DISCUSSION

It is well known that there are marked differences in avian and human malaria, and these need not be repeated here. Nevertheless, the value of work such as that reported in this paper lies in the possibility that it may suggest factors worthy of investigation with human infections. The findings of Seeler and Ott that protein deficiency (Seeler and Ott, '45a) and folic acid deficiency (Seeler and Ott, '45b) and the present demonstration that nicotinic acid deficiency results in more severe infections in avian malaria may be of importance with regard to human malaria. Diets low in protein and nicotinic acid are known to be common in many malarious regions. Recent data indicate that many types of macrocytic anemias respond to folic acid (Anonymous, '46) and thus this deficiency may be not uncommon in the tropics. Assuming that the human malarias respond in a similar manner, these deficiencies might well account for the long suspected relationship between famine and malaria.

Those deficiencies which have been found to inhibit the development of the parasite, i.e., vitamin A and riboflavin (Seeler and Ott, '44), are less likely to be of practical importance. The experimental studies have all been done using acutely deficient birds. Such acute deficiencies are probably uncommon in humans. In this regard it is interesting that the nicotinic acid deficiency in chicks reported here was very mild and produced only a slight decrease in growth, although the change in susceptibility was profound.

Choline deficiency in chicks was not found to increase susceptibility to malaria. It must be borne in mind that this deficiency in chicks does not produce fatty livers nor hemorrhagic kidneys as have been reported in mammalian species.

The studies reported in this paper suggest that the duck is much too susceptible to be a satisfactory experimental animal for studies with *P. lophurae*. No differences were observed

in any of the nutritional deficiencies. It seems likely that the parasite reproduces at near maximum rate in the normal duck and increases in susceptibility, should they occur, would be difficult to detect although Trager ('43) has shown that this is possible. The rapid growth of the animals is also perhaps disadvantageous. As observed in table 4, the birds receiving the adequate ration gained over 100 gm during the course of the infection while the deficient birds gained no weight. Thus there was a marked change in blood volume in the growing birds and although the per cent parasitemia was the same in the two groups, the total multiplication of the parasite was undoubtedly considerably greater in the growing birds. Since the factors underlying the rate of multiplication of the parasite in vivo are unknown, it seems useless to speculate on whether or not the correct evaluation of the studies is the percentage of parasitemia or the total increase in parasites in the bird.

In these studies we have not used starvation or paired-fed controls. As has been pointed out previously (Hegsted et al., '46), starvation and protein deficiencies are in some respects identical since in both instances body tissue cannot be formed or is destroyed. Since protein deficiency (Seeler and Ott, '45) increases the susceptibility to avian malaria, such controls may obscure rather than clarify the results. Seeler and Ott ('44) found in fact in their studies with riboflavin that restriction of food intake had the opposite effect of riboflavin deficiency and increased the severity of the infection. Since various deficiencies, all of which inhibit growth, do not have comparable effects on susceptibility, it appears established that the effects of the nutritional deficiencies in avian malaria are specific.

SUMMARY

The effects of nicotinic acid, thiamine, choline, and vitamin A deficiency on the course of *P. lophurae* infections in chicks and ducklings are reported.

In chicks, vitamin A deficiency appears to cause a somewhat milder infection and choline deficiency may have the opposite

effect. Thiamine deficiency appeared to have no influence on the infection. Nicotinic acid deficiency resulted in a much more severe infection. The percentage of parasitized cells was from four to five times greater in the deficient birds. Both deficient and nondeficient birds were able to clear the blood stream of parasites in approximately the same length of time.

In ducks none of the deficiencies influenced the course of the infection to a marked degree. The duck is probably too susceptible to this infection to be a satisfactory experimental animal.

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EVALUATION OF PROTEINS BY THE NITROGEN BALANCE METHOD¹

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With the ultimate objective in mind of designing a nutritionally efficient mixture of amino acids for intravenous use, preliminary feeding experiments with dogs were undertaken with casein and two grades of fibrin (crude and pharmaceutical³). In some experiments, casein was fortified in various ways with cysteine and methionine. The minimum amount of each protein or protein-amino acid mixture required to maintain nitrogen balance was determined. For the sake of economy of words, it is suggested that this figure be called the MPN (minimum protein nitrogen) value of proteins or amino acid mixtures.

Melnick and Cowgill ('37) have determined the minimum amounts required to maintain nitrogen balance of serum protein, casein, lactalbumin, and gliadin. Their results were calculated on the basis of percentage of calories contributed by the protein when the animal was receiving a total of 70 cal. per kg per day. When their results were recalculated in the same terms used throughout our work, the MPN value for casein was between 217 and 295 mg of nitrogen per kg per day and the MPN value for lactalbumin was between 175 and

¹This material was presented at the American Chemical Society Meeting, April 11, 1946, at Atlantic City.

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200 mg of lactalbumin nitrogen per kg per day. The differences between their work and ours will be brought out in the discussion.

METHODS

Selection and preparation of animals for experimental use

Female dogs in good nutritional condition were selected. To increase the ease of catheterization, an effort was made to use only females which had littered puppies. The animals were weighed and given a vermifuge.

The dogs were placed on a diet supplying an adequate amount of calories (75 cal./kg/day; see composition of the non-protein diet under diets used) and 100 mg of crude fibrin³ nitrogen per kg per day. When casein was used instead of fibrin, 160 mg of casein nitrogen per day were given. This amount of fibrin nitrogen or casein nitrogen was not sufficient to maintain nitrogen balance if the dog was accustomed to a high nitrogen kennel ration. Gradually the negative balance was reduced and in approximately 8 weeks the animal was in positive nitrogen balance and remained there indefinitely. This period of low nitrogen feeding serves to deplete the animal's protein stores to a minimum. The plasma proteins fall to a level below normal and then remain fairly constant. In table 1, the plasma protein data are summarized. Animals prepared in this manner were used for determining the MPN values of proteins and of proteins fortified with amino acids. The data shown in tables 2 and 3 were obtained on animals which had first been subjected to this preliminary depletion period.

The reader will no doubt wonder why 100 mg of fibrin nitrogen per kg per day or 160 mg of casein nitrogen per kg per day were arbitrarily chosen for the amounts of protein nitrogen given during the depletion period. In preliminary experiments the amount of fibrin or casein given to a dog was slowly reduced over a period of a number of weeks until the animal went into definite negative balance. The amounts of

100 mg of fibrin N/kg/day or 160 mg of casein N/kg/day were found to be slightly above the minimum amounts required to maintain nitrogen balance. In order to shorten the depletion period, new animals were started on the experiments at these levels of fibrin or casein nitrogen.

TABLE 1

DOG NO.	WEEKS ON LOW PROTEIN DIET	PERCENT BLOOD ¹ PLASMA PROTEIN	HISTORY OF PROTEIN NITROGEN INTAKE
17	40	4.5	On casein or fibrin intakes supplying minimum or sub-minimum amounts for N balance
	42.5	4.4	100 mg pharmaceutical fibrin N/kg/day
	46.5	4.5	100 mg pharmaceutical fibrin N/kg/day
18	27	4.8	On fibrin intake supplying minimum or sub-minimum amounts for N balance
	29.5	5.4	100 mg pharmaceutical fibrin N/kg/day
	33.5	4.8	100 mg pharmaceutical fibrin N/kg/day
21	19	4.9	On fibrin intakes supplying minimum or sub-minimum amounts for N balance
	21.5	5.4	100 mg pharmaceutical fibrin N/kg/day
	25.5	5.3	100 mg pharmaceutical fibrin N/kg/day

¹ Plasma protein was determined by the method of Drew, Scudder and Papps ('40).

Analysis of urine and feces

Urine was collected under toluene or xylene. It was kept acid with acetic acid to prevent the loss of ammonia.

The dogs were catheterized on the morning of the first day of each period before food was given. Carmine was added to the food of the first day of each period to mark the feces. In the early experiments, the feces were mixed with water and homogenized in a Waring Blender. The mixtures were easily

resuspended before removing samples for analysis. However, it was found that the homogenized feces could be mixed with the urine, thus reducing the number of analyses by one-half. The accuracy of this procedure was checked against separate analyses of the urine and homogenized feces. For analysis, the macro-Kjeldahl digestion procedure was used. A measured portion of the digest was used in a micro-Kjeldahl distillation apparatus.

Diets used

The non-protein portion of the diet consisted of 73 gm sucrose, 20 gm lard,⁴ 3 gm corn oil, 0.5 gm of fish liver oil containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gm, 4.0 gm of U.S.P. salt mixture I, 0.2 gm choline chloride, 1.0 gm agar, 0.6 mg thiamine hydrochloride, 0.6 mg riboflavin, 12 mg nicotinamide, 0.4 mg pyridoxine, 1.2 mg calcium pantothenate, and a liver concentrate low in nitrogen but rich in vitamin B₆. A sufficient amount of this mixture was given to supply 75 cal./kg/day. The nitrogen contained in this amount of the non-protein diet represented usually considerably less than 5% of the total nitrogen intake.

The proteins studied were S.M.A. vitamin-free casein, crude blood fibrin,³ pharmaceutical grade fibrin,³ and lactalbumin.⁵ The amounts of methionine and cysteine used in the fortification of the casein (see table 2) were based on the nitrogen content of the casein rather than on the weight of casein because of the slight variation in the percentage of nitrogen between different lots of this protein.

The kennel weight of each animal was used as the "standard weight" upon which were based the rates of administration of the protein diets and the non-protein supplement. Each day a weighed portion of the protein or protein-amino acid mixture was mixed with the non-protein portion of the diet and fed in the morning. The animals regularly ate all of the food offered each day except for a few dogs when they were re-

⁴ Globe Plankinton.

⁵ Borden.

ceiving extremely low levels of protein intake. In these few cases the laborious procedure of force-feeding the uneaten food each day was avoided. Instead, the entire protein supplement was mixed with only 25 gm of the non-protein diet. This was eaten in a few minutes. The remaining amount of non-protein diet required to supply 75 or 80 cal./kg/day was then placed in the dish. The next morning any unconsumed food left in the dish was weighed.

The hydrolysates

The partial hydrolysate⁶ was prepared by the method of White and Elman ('42). It was concentrated in vacuo to a small volume and lyophilized.

The complete hydrolysate⁶ was made from casein by refluxing 4 days with 3.5 N sulfuric acid. After removal of sulfate ion, the hydrolysate was concentrated in vacuo to a small volume and lyophilized. This material was shown to be substantially completely hydrolyzed $\frac{\text{carboxyl nitrogen (ninhydrin)}}{\text{Total N} - \text{ammonia nitrogen}} = 0.79$.

Tryptophane determinations on supplements E-120c and E-120a were done by the method of Shaw and McFarlane ('38).⁷

RESULTS

Unfortified crude beef blood fibrin³ was found to be one and one-half to two times as effective in maintaining nitrogen balance as unfortified casein. Fortification of casein with cysteine resulted in considerable improvement over unfortified casein. This finding paralleled previously reported experience with rat growth experiments by Osborne and Mendel ('15). Casein fortified heavily with dl-methionine and with cysteine appeared at least equal to unfortified crude fibrin. A purified grade of blood fibrin (pharmaceutical) was slightly less effective in maintaining nitrogen balance than the crude grade.

⁶The author wishes to thank Drs. D. V. Frost and J. R. Schenck of this laboratory for supplying these dried hydrolysates.

⁷The author is indebted to Mr. E. O. Krueger for the tryptophane analyses.

TABLE 2
Summary of the nitrogen balance experiments.

A	B	C	D	E	F
Nitrogen-containing supplement	Protein or protein hydrolysate	Total N ¹ Wt. of dl-methionine added	Total N ¹ Wt. of cysteine HCl · H ₂ O added	Dog no.	Minimum intake which will produce N balance (in mg N/kg/day)
AA11	S.M.A. casein	No fortification		2 17	140-160
AA10	Beef blood fibrin (crude)	No fortification		3 4	70 70
AA10'''	Fibrin, pharmaceutical grade	Not fortified		17 18 21	100 100 80
AA7	S.M.A. casein		4.90	5	100-120
AA8	S.M.A. casein		3.31	6	80-100
AA7a	S.M.A. casein	14.25	4.90	5	80
AA8a	S.M.A. casein	14.25	3.31	6	80
AA9	S.M.A. casein	7.11	4.90	2 4	70 60
E-120c	Incompletely acid-hydrolyzed casein		3.31 ²	4	80
E-120a	Incompletely acid-hydrolyzed casein		4.85 ²	3 4	80 80-100
E-201ds	Completely acid-hydrolyzed casein		4.85 ²	3 4	80 80-100
L-1542	Lactalbumin	Not fortified		10 11	100 ² 100 ²

¹Total N represents the nitrogen of the protein (in gm) before fortification. This is divided by the weight (in gm) of the amino acid used for fortification.

²l-Tryptophane was also added to the dried hydrolysate. The ratio

Total N (in gm) of hydrolysate

Wt. (in gm) of tryptophane present after fortification

was 11.0 for E-120c, 10.0 for E-120a, 12.0 for E-201ds.

³Lower levels of lactalbumin were not given and so this does not necessarily represent the minimum amount of lactalbumin nitrogen required to maintain nitrogen balance. These data were supplied by Dr. Frost of this laboratory.

TABLE 3
Nitrogen balance experiments.

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE
	kg	mg/kg/day	gm N/wk.		kg	mg/kg/day	gm N/wk.
Nitrogen containing supplement: AA11—S.M.A. vitamin-free casein unfortified							
2	5.9	200	+ 1.88	17	9.2	120	— 3.92
	6.0	180	+ 1.21	(continued)	9.3	120	— 0.39
		160	+ 0.56		9.4	140	+ 1.11
	6.0	140	— 0.38		9.8	140	+ 1.25
	5.8	120	— 0.65		10.0	140	+ 0.95
	5.9	140	+ 0.13		10.3	160	+ 0.00
	6.0	160	+ 0.32		10.2	160	+ 2.11
	6.0	142	— 0.63		10.2	140	+ 0.87
	6.0	142	— 0.27		10.4	140	+ 1.43
	6.9	142	— 0.78		10.4	120	— 0.49
	5.9	159	— 1.13		10.4	120	— 0.17
	5.8	159	+ 0.49		10.5	100	— 0.07
17	9.3	140	— 0.13		10.4	100	— 2.03
	9.3	140	— 0.03		10.2	100	— 0.86
	9.0	120	— 0.47		9.9	160	+ 0.57
Nitrogen containing supplement: AA10—Beef blood, fibrin, crude							
4	8.5	100	+ 1.50	3	6.4	60	— 0.90
	8.5	80	— 0.25	(continued)	6.6	50	— 1.12
	8.4	70	+ 0.02	10	8.1	100	+ 1.08
	8.4	60	— 1.08		8.8	100	+ 0.41
3	6.4	125	+ 2.84		8.2	100	+ 0.74
	6.8	100	+ 1.28	11	7.7	100	+ 0.78
	6.6	80	+ 0.47		7.9	100	+ 0.35
	6.7	70	+ 0.07				
Nitrogen containing supplement: AA7—S.M.A. vitamin-free casein + approx. 2.9% of cysteine · HCl · H ₂ O							
5	6.1	80	— 1.67	5	6.3	100	— 0.57
	6.3	83	— 0.96	(continued)	6.3	120	+ 0.81
	6.5	80	— 1.32		6.3	120	— 0.07
	6.3	70	— 1.23		6.4	100	+ 0.30
	6.3	100	— 1.45		6.4	100	+ 0.60
Nitrogen containing supplement: AA8—S.M.A. vitamin-free casein + approx. 4.3% of cysteine · HCl · H ₂ O							
6	10.0	80	+ 0.21	6	10.3	76	— 0.70
	9.9	81	— 0.54	(continued)	10.2	100	+ 0.55
	10.3	70	— 2.31		10.2	100	+ 0.56
	10.2	60	— 1.72		10.3	100	+ 1.04
	10.2	80	— 1.15		10.5	80	— 0.37

TABLE 3 (continued)

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE
	kg	mg/kg/day	gm N/wk.		kg	mg/kg/day	gm N/wk.
Nitrogen containing supplement: AA7a—S.M.A. vitamin-free casein + approx. 1% of dl-methionine + 2.9% of cysteine · HCl · H ₂ O							
5	6.5	78	—0.87	5	6.4	84	+0.41
	6.7	68	—0.86	(continued)	6.4	83	+0.18
	6.3	70	—0.73		6.5	80	+1.29
	6.4	70	—1.37		6.4	84	+0.65
	6.5	80	—0.29				
Nitrogen containing supplement: AA8a—S.M.A. vitamin-free casein + approx. 1% of dl-methionine + 4.3% of cysteine · HCl · H ₂ O							
6	10.5	79	+0.06	6	10.5	86	+1.63
	10.5	69	—0.73	(continued)	10.5	85	+0.99
	10.5	70	—0.58		10.2	80	+2.46
	10.5	70	—0.50		10.5	80	+1.87
	10.5	80	+0.35				
Nitrogen containing supplement: AA9—S.M.A. vitamin-free casein + approx. 2% of dl-methionine + 2.9% of cysteine · HCl · H ₂ O							
2	6.1	70	+0.66	2	6.3	70	—0.50
	6.1	70	Urine sample lost	(continued)	6.3	70	+0.25
	6.1	60	—0.55	4	8.4	81	+1.53
	6.1	50	—0.63		8.4	70	+0.74
	6.4	70	+0.47		8.5	60	+0.09
			(9-day period)				
Nitrogen containing supplement: E-120c—Dried partial acid hydrolysate of casein + approx. 4.3% cysteine · HCl · H ₂ O							
4	8.4	80	+0.50	4	8.4	50	—0.51
	8.4	60	—0.38	(continued)	8.0	50	—0.53
Nitrogen containing supplement: E-120a—Dried partial acid hydrolysate of casein + approx. 2.9% cysteine · HCl · H ₂ O							
3	7.2	100	+0.90	4	8.4	80	—0.23
	7.2	80	+0.33		8.4	60	—1.30
	7.1	60	—0.65				

TABLE 3 (continued)

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED ¹	NITROGEN BALANCE
	kg	mg/kg/day	gm N/wk.		kg	mg/kg/day	gm N/wk.
Nitrogen containing supplement: E-201ds—Complete acid hydrolysate of casein + approx. 2.9% cysteine · HCl · H ₂ O							
3	6.9	100	+ 0.82 (6-day period)	4	7.6	80	— 0.64
	6.9	80	+ 0.07 (8-day period)		7.9	64	— 0.59
	6.8	60	Negative		8.2	48	— 1.81
Nitrogen containing supplement: L-1512—Lactalbumin							
10	8.4	100	+ 0.84	11	7.7	100	— 0.76
	8.7	100	+ 0.79		8.4	100	+ 0.42
	8.3	100	+ 1.17		7.7	100	+ 1.13
Nitrogen containing supplement: AA10 ^{'''} —Beef blood fibrin, pharmaceutical grade							
17	9.0	79	— 3.21	18	7.5	80	— 0.24
	9.2	80	— 3.39	(continued)	7.5	80	— 0.09
	9.0	100	+ 0.23		7.5	100	+ 0.78
	9.0	80	— 2.14		7.5	100	+ 0.96
	9.0	80	— 0.89		7.5	100	+ 0.76
	8.8	80	— 0.71	21	9.5	79	— 0.09
	8.6	100	— 0.06		9.4	80	— 0.28
	8.5	100	+ 0.15		9.3	100	+ 1.53
	8.4	100	— 0.53		9.5	80	+ 0.58
18	7.6	61	— 2.18		9.4	80	+ 0.77
	7.6	79	— 0.94		9.4	80	+ 1.14
	7.6	80	— 0.62		9.4	100	+ 2.06
	7.6	100	+ 0.13		9.7	100	+ 2.71
	7.5	80	— 0.29		9.7	100	+ 2.33

¹ These figures were calculated from the "standard" or kennel weights of the dogs. The standard weights in kilograms of the dogs used are listed as follows: Dog 2, 6.9; Dog 3, 6.6; Dog 4, 8.4; Dog 5, 7.2; Dog 6, 10.0; Dog 10, 8.1; Dog 11, 7.5; Dog 17, 9.1; Dog 18, 7.2; Dog 21, 9.8.

In table 2 the results of the nitrogen balance experiments are summarized. In table 3 the results of the weekly nitrogen balance determinations are recorded. For each animal, these results appear in the order in which the experiments were carried out.

DISCUSSION

The literature on the protein minimum prior to 1937 has been adequately reviewed by Melnick and Cowgill ('37).

It is important to note that the animals we used for determining the protein minimum were in a protein depleted state, having gone through an adjustment period of 2 months to the low nitrogen intake of 100 mg of fibrin nitrogen per kg per day or 160 mg of casein nitrogen per kg per day. Their plasma protein levels were in the range of 5%. In the experiments of Melnick and Cowgill a 3- to 7-day period was used to allow the animals to adjust their metabolism to a new dietary regime. The level of nitrogen fed during the short adjustment period was never lower than 160 mg of casein nitrogen per kg per day or 150 mg of lactalbumin nitrogen per kg per day. The blood protein levels and general protein stores of the animals after this adjustment period were probably very nearly normal. Therefore, the protein minimums determined by Melnick and Cowgill were for dogs with normal protein storage supplies. The protein minimums we have determined are very close to the absolute minimums for survival when the animal receives adequate calories from carbohydrate and fat. If an animal is fed for prolonged periods of time less than the minimum levels of protein we have established, the animal's health deteriorates badly.

Although the protein minima for nitrogen equilibrium determined by the technique of Melnick and Cowgill are different from those we determined, their methods give results very similar to ours when one protein is compared to another. For example, taking the figures of Melnick and Cowgill and giving lactalbumin an arbitrary nutritional value of 100, the value found for casein was seventy-three. According to our findings, if 100 mg of nitrogen per kg per day is assumed to be the minimum amount of lactalbumin for nitrogen equilibrium (the minimum amount is probably very close to this), the relative nutritional value of casein is $\frac{100}{150} \times 100$ or 66.6.

From the cystine and methionine contents of casein and fibrin reported in the literature (Block and Bolling, '45),

(fibrin contains 1.5% cystine, 2.2% methionine; casein contains 0.3–0.5% cystine, 3.0–3.3% methionine) it is evident that the total content of sulfur amino acids in the two proteins is approximately the same. Yet, as described in the preceding paragraphs, we have found that casein is inferior to fibrin until it is fortified with cysteine and methionine whereupon it becomes equal to fibrin in ability to maintain nitrogen balance.

A possible explanation for this is that the cystine and methionine of casein are not well utilized, perhaps due to incomplete hydrolysis of the protein in the digestive tract. To test this point, the MPN value of a dried complete hydrolysate of casein fortified with only tryptophane should be determined and compared to that of unfortified casein. This has not been done, but a comparison of preparations E-120a and E-201ds (table 2), which are dried hydrolysates of casein fortified with tryptophane and cysteine, with AA7 (table 2), which is unhydrolyzed casein fortified with the same amount of cysteine, shows that the hydrolyzed preparations were slightly more effective in maintaining nitrogen balance. The experimental evidence on this point remains inconclusive however, because the hydrolysates were fortified with cysteine.⁸

It is claimed by Miller ('44) that cystine and methionine have a protein sparing action when added to diets very low in protein. Some such action may contribute to the greater effectiveness of casein fortified with cysteine and methionine in maintaining nitrogen balance.

Casein contains somewhat smaller quantities of threonine, tryptophane, phenylalanine, and leucine than does fibrin.

⁸ After this paper was submitted for publication, Melnick, Oser, and Weiss (Science, vol. 103, p. 326, 1946) produced evidence that in vitro the enzymatic release of methionine from raw soy bean meal is slower than from heat-processed soy bean meal. The decreased nutritional value of raw soy bean meal would then be due to the late absorption of methionine. The same may be true of the sulfur amino acids of casein, thus providing another possible explanation for the beneficial effect of supplementing casein with methionine or cystine or both.

Since fortification of casein with adequate amounts of cysteine and methionine results in a mixture equally effective as fibrin in maintaining nitrogen balance, it is unlikely that casein is inferior to fibrin because it contains insufficient amounts of threonine, tryptophane, phenylalanine, or leucine.

It is not probable that streptogenin, the peptide-like growth factor for bacteria and mice, described by Sprince and Wooley ('44, '45, '45), played a role in these nitrogen balance studies. It is destroyed by acid hydrolysis of casein and it can be seen in table 2 that casein hydrolysates E-120a and E-201ds were slightly more effective in maintaining nitrogen balance than unhydrolyzed casein similarly fortified.

SUMMARY

By employing suitably prepared dogs, the minimum amount of a protein required to maintain nitrogen balance (MPN value) was determined.

The MPN values of casein, crude fibrin, and pharmaceutical grade fibrin were found to be, respectively, 140-160, 70 and 80-100 mg N/kg/day.

Fortification of casein with cysteine reduced the MPN value to 80-100 mg N/kg/day. When both cysteine and methionine were added to casein in sufficient amounts, the MPN value fell to 60-70 mg N/kg/day.

The MPN value for lactalbumin was not determined, but it maintained nitrogen balance when given at the rate of 100 mg of lactalbumin nitrogen/kg/day.

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INTRAVENOUS UTILIZATION OF PARTIAL ACID HYDROLYSATES OF PROTEINS¹

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Investigations dealing with the efficacy of protein hydrolysates in intravenous nutrition have been reviewed by Elman ('44). Although no strictly quantitative data are available, there are indications that the peptide component of an enzymatic hydrolysate is utilized for anabolic processes. However, it is generally recognized that there is no definite proof of this Sahyun, '44).

White and Elman ('42) hydrolyzed casein and pumpkin seed globulin with sulfuric acid under mild conditions. Hydrolysis was incomplete but the major portion of the tryptophane remained intact under the conditions employed. When dried and fed to dogs, these hydrolysates maintained nitrogen balance. Recently, White and Sayers ('45) have reported further studies on incompletely acid-hydrolyzed proteins. These hydrolysates were injected rapidly into the veins of rats, guinea pigs, rabbits and dogs with no undesirable reactions resulting. No nitrogen balance studies were made however. Preparations of incompletely acid-hydrolyzed proteins will be called partial acid hydrolysates.

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By means of nitrogen balance studies in dogs, we have sought to determine whether the peptide component of a partial acid hydrolysate of a protein is utilized when the hydrolysate is administered by the intravenous route. This was done by determining the minimum amounts of complete and partial hydrolysates required to maintain nitrogen balance. A brief report covering part of this work has already been presented (Risser, Schenck and Frost, '46).

METHODS

The selection of animals, the preparation of animals for the nitrogen balance experiments, and the collection and analysis of urine and feces were done exactly as described previously (Risser, '46).

The complete hydrolysates were prepared by refluxing casein 4 days with 3.5 N sulfuric acid. The sulfate-free hydrolysate was shown to be substantially completely hydrolyzed,

$$\frac{\text{carboxyl nitrogen (ninhydrin)}}{\text{total nitrogen} - \text{ammonia nitrogen}} = 0.79.$$

The partial acid hydrolysates were prepared by the method of White and Elman ('42) except that shorter refluxing periods were used (2 hours and 4 hours) in some experiments. The per cent of free amino acid nitrogen in these partial hydrolysates has been determined by Frost and Heinsen ('45).

Fortification of sulfate-free partial and complete hydrolysates with amino acids was based on a Kjeldahl nitrogen determination on the solution. The amino acids were added, the solutions were diluted to a volume convenient for use (5-7 mg N/ml) with pyrogen-free water, bottled, and quickly sterilized. The solutions were tested for pyrogens and another Kjeldahl nitrogen determination was obtained. The amounts of hydrolysate injected per day were based on this last Kjeldahl nitrogen determination (see seventh column of table 1).

The various types of fortifications with amino acids are given in table 1. This method of expressing the amounts of amino acids added to the hydrolysates may appear somewhat unusual, but it was used for the following reasons. The

amounts of amino acids were based on the nitrogen content rather than on the solid content of the hydrolysates because the percentage of total nitrogen varied slightly in different batches of the hydrolysates. The only accurate method of duplicating the amounts of amino acids added to new batches of fortified hydrolysate then was to base these additions on the nitrogen content. Likewise, since the amounts of the hydrolysates injected were expressed in milligrams of nitrogen per kilo per day, a better duplication of the amounts of fortifying amino acids administered per day was effected.

The amounts of amino acids used in fortifying can be approximately expressed in per cent of solids in the hydrolysate by assuming a total nitrogen of 14.5% for casein hydrolysates and a total nitrogen of 13.5% for fibrin hydrolysates. If Total N is divided by the ratios in the third and fourth columns of table 1, the result gives the percentage fortification. For example, hydrolysate E-214 contained $\frac{14.5}{12} = 1.2\%$ dl-tryptophane and was fortified with $\frac{14.5}{4.90} = 2.9\%$ cysteine monohydrochloride monohydrate.

The non-nitrogenous portion of the diet has been described (Risser, '46). A sufficient amount of this diet was given each day to provide 75 cal./kg/day. Daily records were kept of the amount of this diet consumed. The variation that occurred in the intake of this diet did not reflect in unusual weight changes (determined each week) in the animals and the amount consumed did not fall to levels which would be expected to affect the nitrogen balance results. The nitrogen contained in this portion of the diet amounted to less than 5% of the nitrogen provided by the intravenous hydrolysates.

For the intravenous injection of the hydrolysates each animal was fastened securely in an upright position to a frame designed especially for these experiments. The solution of the hydrolysate was poured into a sterilized burette (50-, 100-, or 250-ml capacity, depending upon the volume of solution to be given). The burette was equipped with a rubber tube, clamp and 20-gauge needle.

The needle was introduced into a leg vein. It was held in place with a strip of tape. The proper rate of flow of solution into the vein was obtained by regulating the clamp on the rubber tube and by adjusting the height of the burette. During the injection, the burette was periodically raised to keep the flow constant. The hydrolysates were given daily in one injection. This injection period in most of the experiments was 80 or 120 minutes (see table 1). With proper rotation of the use of the leg veins, it was possible to keep a dog continuously for 3 months on intravenous amino acids before the veins became difficult to use.

RESULTS

Solutions of completely hydrolyzed casein fortified with tryptophane and cysteine monohydrochloride monohydrate (solutions E-214 and E-215, table 1) were given intravenously to female dogs. The minimum intake of nitrogen which maintained nitrogen balance was found to be approximately 120 mg/kg/day.

Partial hydrolysates of casein (solutions E-185a and E-245 in table 1) were fortified with approximately the same amounts of cysteine as used in complete hydrolysates E-214 and E-215. Nitrogen balance was produced at an intake of 120 mg N/kg/day. Thus, although three-fourths of the amino acids present were bound in peptides (Frost and Heinsen, '45), they were available to the animal for the purpose of maintaining nitrogen balance.

Although hydrolysate E-185a was fortified with tryptophane to the same content of this amino acid in unhydrolyzed casein, this solution was no more effective than hydrolysate E-245 (table 1) which contained only the tryptophane left undestroyed by the hydrolysis. However, when the partial acid hydrolysate was not fortified with cysteine (solutions E-293 and E-298 in table 1), it was not as effective in maintaining nitrogen balance as the cysteine fortified hydrolysates.

A partial hydrolysate of fibrin was prepared using the conditions of White and Elman ('42). The amount of tryptophane

TABLE 1
Summary of nitrogen balance experiments.

HYDROLY- SATE NO.	HYDROLYSATE	TOTAL N ¹ WEIGHT OF TRYPTOPHANE PRESENT ²	TOTAL N ¹ WEIGHT OF CYSTEINE HCl. H ₂ O ADDED	DOO NO.	INJECTION PERIOD (MINUTES)	INTAKE OF HYDRO- LYSATE IN MG N/KG/DAY	BALANCE IN GM N/WEEK
E-214	casein, complete	12.0 (dl-trypto- phane added)	4.90	1	80 80 80	100 80 120	-0.94 -0.99 -0.49
E-215	casein, complete	12.0 (dl-trypto- phane added)	3.31	2	80 80 80	100 80 120	-1.33 -0.99 +0.26
E-185a	casein, partial (6 hr.)	11.0 (dl-trypto- phane added)	4.83	1	80 80 80	120 100 80	+0.33 -0.38 -0.62
				2	80 80	120 140	-0.14 +0.54
E-245	casein, partial (6 hr.)	25.0 (no trypto- phane added)	4.83	3	80 80 100 90	140 140 120 100	+0.78 +0.20 +0.14 -0.05
				3	120 120	140 140	-0.97 -0.48
E-293	casein, partial (6 hr.)	23.8 (no trypto- phane added)	no cysteine added	4	120 120 120 120	140 140 121 120	-1.17 -1.90 -1.39 -1.68
E-298	partial (6 hr.) of 1 part fibrin, 3 parts casein	18.4 (no trypto- phane added)	no cysteine added	7	120 120 120	161 140 140	-0.40 -0.34 -0.73
				8	120 120 120	148 120 120	-0.51 -0.95 -1.69
E-270	crude fibrin, partial (6 hr.)	9.0 (no trypto- phane added)	14.06	4	120 120	100 100	+1.02 -0.13
E-2B	fibrin, partial (2 hr.)	9.63 (no trypto- phane added)	7.63	3	120	100	-0.41
				7	120	120	+0.71
C-2B	casein, partial (2 hr.)	18.1 (no trypto- phane added)	6.65	4	120 120	120 120	+0.61 +0.95
F-4B	fibrin, partial (4 hr.)	7.42 (no trypto- phane added)	5.75	4	120 120 120	120 130 100	+0.77 +0.60 +1.17
C-4B	casein, partial (4 hr.)	18.6 (no trypto- phane added)	6.58	3	120 120 120	120 120 140	+0.45 +0.34 -0.02
				7	120 120	140 140	+0.27
E-216	casein, complete	12.0 (dl-trypto- phane added)	4.90 dl-meth- ionine was also added.	4	120 120 120 120	120 100 100 80	+0.71 +0.34 +0.78 -0.16
<i>Total N = 14.2</i>							
<i>dl-methionine added</i>							
E-201s	casein, complete	13.14 (dl-trypto- phane added)	14.50 this ratio is for dl- methionine added. No cysteine added	1	80	120	-0.15
				2	80 80	120 100	-0.14 -1.44

¹ In this ratio, Total N represents nitrogen (in gm) of the hydrolysate before any additions of amino acids were made. Also, under Methods, see Fortification.
² For partial hydrolysates were determined. "Tryptophane of the tryptophane retained in the partial hydrolysate of the hydrolysates, it represents the amount added."

left intact was much greater than in the casein hydrolysates similarly prepared.³ The hydrolysate, fortified with cysteine (solution E-270, table 1), maintained nitrogen balance when given at the rate of 100 mg N/kg/day although over 60% of the amino acids were found in peptides (Frost and Heinsen, '45).

Both casein and crude fibrin were hydrolyzed by the method of White and Elman ('42) except that refluxing periods of 2 hours and 4 hours were used. Each hydrolysate was fortified with approximately the same amount of cysteine (see solutions F-2B, C-2B, F-4B, and C-4B, table 1). All of these hydrolysates were as effective as the 6-hour hydrolysates in maintaining nitrogen balance. The 2-hour fibrin and casein hydrolysates contained 20 and 17% free amino acids, respectively. Similar figures for the 4-hour fibrin and casein hydrolysates were, respectively, 33 and 20% (Frost and Heinsen, '45).

Complete casein hydrolysates E-216 and E-201s (table 1) represent different types of fortification with cysteine and methionine than were used in any of the partial hydrolysates studied. However, the results with these solutions further substantiate the fact that the minimum amount of a complete hydrolysate given intravenously capable of maintaining nitrogen balance is in the range of 100-120 mg N/kg/day.

Excretion of amino acids and peptides in the urine

We have not made a thorough study of the excretion of amino acids and peptides following the injection of these hydrolysates. However, in using the method of Van Slyke, MacFadyen and Hamilton ('43), we have found that after oral administration of fibrin or casein at the rate of 100 to 130 mg N/kg/day, 3 to 9% of the total nitrogen excreted was in the form of amino acid nitrogen. When the urine was hydrolyzed with 8 N H₂SO₄ for 18 hours, amino acid nitrogen was approxi-

³ We wish to thank Mr. Elmer Krueger for all the tryptophane determinations appearing in this paper. They were done by the method of Shaw and McFarlane ('38).

mately doubled. Partial (6 hours) fibrin hydrolysates given intravenously at the rate of 120 mg N/kg/day in a 2-hour period gave approximately the same picture in amino acid and peptide excretion. When the hydrolysate was given in a much shorter period of time, there was a detectable increase in excretion of amino acids and peptides. For example, one dog received intravenously a partial hydrolysate of casein (6 hours) at the rate of 162 mg N/kg/day in 35 minutes. In the urine, 15.5% of the total nitrogen was found to be amino acid nitrogen. After hydrolysis with sulfuric acid, free amino acid nitrogen was found to be 28.7% of the total nitrogen.

Whether or not the extra amino acid nitrogen found after acid hydrolysis of urine actually originated from peptides we have not further investigated.

Reactions to these solutions, such as vomiting, were very infrequent, especially when the 2-hour injection period was used. Since the injections were made at a time when the animal's stomach was empty, the vomitus was in the form of mucus. This was mixed thoroughly with the urine for analysis. There was considerable variation in tendencies for different dogs to vomit.

DISCUSSION

Holman et al. ('34), Pommerenke et al. ('35), and Daft et al. ('38) have shown that a dog can be maintained in nitrogen balance when intravenously injected dog plasma is the only source of protein. Horse plasma protein was not similarly utilized. The mechanism of utilization of plasma protein for synthesis of other tissue protein was thought to involve cleavage of the plasma protein to large aggregates rather than to amino acids (Whipple, '38). It was postulated that these aggregates or peptides were used as building blocks to form specific cell protein or reserve store protein.

We believe that it is unlikely that the peptides of a partial acid hydrolysate are incorporated as such into tissue protein. It seems reasonable that they must be hydrolyzed to amino acids and that specific cell protein could be synthesized only from these amino acids. The literature shows that there are

polypeptides present in normal blood (Hiller and Van Slyke, '22; Godfried, '39; London and Kotschneff, '34) and polypeptidases (Grassmann and Heyde, '30; Abderhalden and Hanson, '37; Sennhenn, '41). Furthermore, it is reported that parenteral injection of peptides results in an increase of peptidase in plasma and serum (Hanson, '34). In the light of this information, it is not surprising to find that the peptides of partial acid hydrolysates are utilized by the dog after intravenous injection.

SUMMARY

We have determined the minimum amount required to maintain nitrogen balance of a complete acid hydrolysate of casein fortified with tryptophane and cysteine after intravenous injection into female dogs. The same was done for a partial acid hydrolysate of casein similarly fortified. Although 83 to 75% of the amino acids of the partial hydrolysate were bound in peptides, this partial hydrolysate was just as effective in maintaining nitrogen balance as was the complete hydrolysate given at the minimum level (120 mg N/kg/day), which would maintain nitrogen balance. This provides excellent evidence that the peptides of the partial acid hydrolysate are utilized for anabolic processes.

Partial and complete casein hydrolysates and partial fibrin hydrolysates were fortified in different ways with tryptophane, cysteine, and methionine and the effectiveness was studied of each fortified hydrolysate in maintaining nitrogen balance after intravenous administration.

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PROTEIN DEPRIVATION AS A CAUSE OF VASCULARIZATION OF THE CORNEA IN THE RAT

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TWO PLATES (TWELVE FIGURES)

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The various effects of protein deprivation or deficiency have been summarized by Metcalf, Favour and Stare ('45). We have recently reported (Sydenstricker, Hall, Hock and Pund, '46) that the appearance of corneal vessels in the rat may also be a result of protein deficiency, as well as of a deficiency of certain amino acids. While it is possible that corneal vascularization may result in man from inadequate protein nutrition, on biomicroscopic examination of some twenty malnourished individuals, all suffering from nutritional edema, none showed corneal vascularization. Lyle, McCrae and Gardiner ('44) found that the addition of about 230 gm of liver and 115 gm of kidney per week to the diet of eighty-two airmen for 10 weeks caused no significant improvement in the degree of corneal vascularization. Little corneal vascularity had been observed where the food was of good nutritive value but more was found where the food was less satisfactory.

Our present paper is an extension and amplification of our previous report on the occurrence of corneal vessels in rats suffering from a deficiency of protein (Sydenstricker, Hall, Hock and Pund, '46).

EXPERIMENTAL

Twenty-three rats of a Wistar strain were placed on a protein-free diet (diet 29, table 1) when from 50 to 62 days of age and thirteen rats from the same litters were fed the control diet (diet 31, table 1). We had previously observed that four rats placed on diet 29 when 30 days of age died before any very marked eye abnormalities could be seen with the biomicroscope (Sydenstricker, Hall, Hock and Pund, '46). Two out of three rats placed on the protein-free diet when 39 days of age had developed corneal vessels. The eyes of the rats

TABLE 1

Constitution of the protein-free diet (29) and control diet (31).

	DIET 29 PROTEIN FREE	DIET 31 CONTROL
Vitamin free casein (Labco)		240 gm
Sucrose	908 gm	668 gm
Salt mixture ¹	40 gm	40 gm
Cottonseed oil	30 gm	30 gm
Cod liver oil, U.S.P.	20 gm	20 gm
Choline chloride	2 gm	2 gm
Thiamine chloride	4 mg	4 mg
Pyridoxine hydrochloride	4 mg	4 mg
Riboflavin	16 mg	16 mg
Calcium pantothenate	20 mg	20 mg

¹ The salt mixture was as used by McKibbin, Madden, Black and Elvehjem ('39).

on the protein-free diet were examined every other day with the biomicroscope and the eyes of the group of control rats were examined weekly. During these examinations rats with typical eye changes were selected for injection or for studies of histological changes. The various techniques employed and the procedure in caring for the animals were as previously described (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

In order to follow the regression of corneal vessels produced in rats fed the protein-free diet (diet 29), ten rats with varying degrees of vascularization were changed from the protein-

free diet to the control diet (diet 31). Resulting changes in the cornea were then followed with the biomicroscope and typical specimens were injected or taken for histological studies.

Three male rats 7 months of age were placed on the protein-free diet in order to determine the length of time necessary for corneal vessels to appear in the adult rat. Biomicroscopic examination of the eyes of these rats was made at weekly intervals.

In order to observe any relationship which might exist between the appearance of corneal vessels and the level of total plasma protein and blood hemoglobin, twenty rats were placed on the protein-free diet (diet 29) and seventeen rats from the same litters were placed on the control diet (diet 31). Blood samples for hemoglobin and protein determination were obtained by ventricular puncture from a sufficient number of rats in each group to provide a fair sampling of the group. The first sample was taken at the beginning of the experiment, a second, after corneal vascularization had appeared in most of the deficient rats, and a third after the deficient rats had been placed on the control diet and the vessels had regressed. Hemoglobin and total plasma proteins were determined by the methods of Evelyn ('36) and of Johnston and Gibson ('38), respectively. In the determination of total plasma proteins, the tyrosine factor for human blood was used, since Greenberg ('37) had found this factor for the rat and for man to be practically identical. The results of these determinations are summarized in table 2.

All the experimental animals were given food and water ad libitum and were kept in cages with wire bottoms, usually one or two animals to a cage. The rats were fed daily and weighed semiweekly.

In this investigation we have adhered to the practice which we earlier adopted (Bowles, Allen, Sydenstricker, Hock and Hall, '46) of limiting our use of the term "corneal vascularization" to the situation where the extent of corneal capillary invasion exceeds the limits of normal variation. These limits

TABLE 2

Average total plasma protein and blood hemoglobin levels in rats on deficient and control diets. All values are in gm per 100 ml.

	CONTROL RATS DIET 31		DEFICIENT RATS DIET 29	
	Hemoglobin	Plasma protein	Hemoglobin	Plasma protein
At beginning of experiment	13.28 ± 0.54 (13) ¹	5.67 ± 0.20 (13)	13.23 ± 0.39 (19)	5.47 ± 0.18 (19)
At time of ap- pearance of vascularization	14.91 ± 0.53 (10)	5.86 ± 0.11 (11)	12.34 ± 0.57 (10)	4.93 ± 0.19 (9)
After vasculari- zation had re- gressed through feeding diet 31 to deficient rats	14.80 ± 0.29 (9)	6.14 ± 0.19 (8)	12.98 ± 0.73 (7)	6.08 ± 0.25 (7)

¹ Numbers in parentheses indicate the number of rats on which the determinations were made.

we determined by examining the eyes of some 500 normal rats.

RESULTS AND DISCUSSION

The ocular changes observed in rats on the protein-free diet were not entirely uniform and seemed to depend to some extent on the size and age of different litters of rats and, at times, to differences among litter-mates.

In the majority, the first definite change was edema of the scleral conjunctiva; often this was marked; sometimes it was not observed. Usually a day or two later there was congestion of the conjunctiva with engorgement of the circumferential vessels of the limbic area. Often there was a slight and usually transient thickening and opacity of the cornea. This may have been due to edema alone or to edema plus leukocytic infiltration. In a few animals corneal opacity was striking and nebulae were seen in addition to diffuse opacity. Whether or not corneal opacity was observed, in about a week,

sometimes as soon as 2 days after the development of conjunctival congestion, there was evidence of beginning invasion of the cornea by new-formed capillaries.

Frequently these capillaries stemmed from the circumferential vessels opposite the points where feeder vessels were visible; almost as often "sprouts" from the circumferential artery appeared at numerous points widely separated from the "feeders." There was no uniformity in the process of corneal vascularization. In the majority of rats a dozen or more capillaries sprouted from the circumferential artery and rapidly invaded the superficial area of the cornea. These capillaries seemed to lie immediately beneath the corneal epithelium and, as they grew larger, often produced ridges on the surface of the cornea. Eventually, they reached the center of the cornea and anastomosed with capillaries invading from the opposite side. In a few instances the vessels assumed a dendritic appearance with clusters of capillary loops terminating the invading vessel. In these rats a rather dense collar of vessels eventually was formed extending about half-way from the limbus to the center of the cornea. The peak of corneal vascularization seemed to be reached some 2 to 3 weeks after the first evidence of ocular involvement. After this, in rats continued on the protein-deficient diet, there was a period of 2 to 3 weeks during which corneal opacity recurred, sometimes as diffuse cloudiness, sometimes as localized nebulac.

Treatment with the control diet (diet 31) produced rapid regression of corneal vascularization. The new-formed vessels in the cornea grew narrower, there was "beading" of the blood columns in them, and eventually the vessels became invisible or were represented by fine white streaks in the cornea, probably the endothelial columns persisting after patent capillaries ceased to be visible. Concurrently such opacity as existed cleared up, leaving a perfectly transparent cornea. As might have been expected, narrowing of the invading capillaries and eventual disappearance was much more rapid when treatment was begun before the ultimate grade of vas-

cularization was reached. In some animals a few large anastomosing vessels have persisted for weeks after all the network of finer capillaries had become invisible.

Figures 1 to 8 are anterior and oblique views of injected corneas from four rats, which show different degrees of the development of corneal vascularization due to protein deprivation. It was noted in corneal preparations from rats suffering from protein deprivation that the newer portion of the injected capillary near the center of the cornea appeared larger in diameter than the rest of the capillary. This was in contrast to what was observed in vitamin A and riboflavin deficiencies where the newer portion of the injected capillary tended to be smaller. Since in all cases the injection pressure was approximately the same, this may mean that the newer portion of the capillary is more readily distensible, or on the other hand it may be merely an indication of the increasing severity of the deficiency during the development of the capillary. Once invasion of the cornea begins, the time required for the capillaries to penetrate nearly to the center of the cornea is roughly the same in vitamin A, riboflavin or protein deficiencies. However, the period of previous deficiency before invasion begins is long in vitamin A and riboflavin deficiencies, while in protein deprivation this period is rather short and almost equals the time required for extensive invasion. The degree of deficiency of the animal is probably increasing much more rapidly during the corneal capillary invasion in protein deprivation than with the vitamin A and riboflavin deficient rats.

The suggestions which we made in an earlier paper (Bowles, Allen, Sydenstricker, Hock and Hall, '46) as to the pattern of vascularity in riboflavin and vitamin A deficiencies depending on the progress of the capillary invasion of the cornea and the development of capillary branches, seem to apply also in the vascularization resulting from protein deprivation. The general pattern of the vessels as observed in each of these three deficiencies appears to be very similar, though there is some difference in detail. We also have observed in these prep-

arations the tendency of the capillaries to arch over the points where the feeder vessels bifurcate to form the circumferential vessels as we found in vitamin A and riboflavin deficiencies. In figures 5 and 7 it may be observed that vascularization has proceeded more rapidly in two quadrants than in the other two. This also is in accord with our observations with other deficiencies. Showing faintly in the photographs used for figures 7 and 8 were small vessels anastomosing over the center of the cornea. Since the injection fluid did not enter these vessels, it may be that they were not yet functional.

Figures 9, 10, 11 and 12 show three stages in the regression of corneal vessels which resulted when the deficient rats were changed to the control diet. At the time the injection preparation shown in figures 9 and 10 was made, the vessels had diminished in size and were bloodless, but the injection pressure was sufficient to force the ink into the vessels as far as they had been observed to extend on biomicroscopic examination. The cornea shown in figure 11 developed vessels extending two-thirds of the way to the center while on the deficient diet. At the time of injection these were also much diminished in size and bloodless. As may be seen, the vessels were opened to half of their length by the injection fluid. Figure 12 shows a cornea which had been vascularized nearly to the center and was similar in degree to the vascularization shown in the cornea in figures 7 and 8. At the time of injection the vessels were bloodless and were no longer visible with the biomicroscope. It may be seen, however, that the injection fluid penetrated a small portion of the proximal end of some of the capillaries which were much diminished in size. A further part of some of the capillaries showed faintly in the photograph even though not filled with injection fluid.

A discussion of the changes in histology of the cornea resulting from protein deprivation will be included in a later paper.

In the rats which were 50 to 62 days of age, corneal vessels appeared in all the rats on the protein-free diet in from 9 to 20 days. Corneal vessels developed in the three adult rats

at 24 days, 38 days and 59 days, respectively. Four rats which had been placed on the protein-free diet when 30 days of age died without developing corneal vessels (Sydenstricker, Hall, Hock and Pund, '46). It appears that the age of the rat when placed on the diet and the degree of deficiency are important factors in determining whether or not corneal vascularization results, as well as the length of time required for corneal vascularization to develop, just as with all other nutritional deficiencies so far studied (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

In the course of the biomicroscopic examinations of the rats' eyes it became evident that the animals suffered from a very definite photophobia which appeared approximately at the time when significant eye changes were first observed. We (Bowles, Allen, Sydenstricker, Hock and Hall, '46) had observed a similar photophobia in rats suffering from vitamin A or riboflavin deficiency as had other investigators.

None of the thirty rats on the control diet (diet 31) showed more than normal variation in the width of the limbic area and in none of these was corneal vascularization ever observed. Since that time, we have had thirty other rats on this control diet, some for as long as 4 months, of which only one developed a mild degree of corneal vascularization.

Rats on the control diet (diet 31) grew an average of 2.2 gm per day as compared with 2.4 gm per day for a group of nine rats of the same strain and age which were on a diet of a commercial dog chow,¹ lettuce, carrots and milk. The rats on the protein-deficient diet (diet 29) lost an average of 1.3 gm of weight per day.

It was observed that many of the rats on the control diet (diet 31) developed a rusty, disheveled appearance and, in some cases, shed part of their hair. These rats were individually fed a daily supplement supplying 1 mg per day each of inositol, para-aminobenzoic acid and nicotinic acid. Within 10 days the rats had resumed a normal appearance. One mg daily of riboflavin, or of nicotinic acid alone had no such ef-

¹ Purina.

feet. Control rats in other experiments with similar diets, where the carbohydrate was supplied as starch rather than the sucrose used here, never developed the disheveled appearance described above. The significance of these observations is not entirely clear, though the explanation probably involves intestinal synthesis of substances important to the nutrition of the rat by the intestinal flora. Mannering, Orsini, and Elvehjem ('44) found that with rats on a diet suboptimal with respect to riboflavin, sucrose minimized growth and fecal excretion of riboflavin as compared with starch or dextrin as a source of carbohydrate, indicating that the starch favored intestinal synthesis of the vitamin while sucrose minimized this effect. Similarly, McIntire, Henderson, Schweigert and Elvehjem ('43) ascertained that the growth of rats on a synthetic diet containing limiting amounts of thiamine was stimulated by the administration of para-aminobenzoic acid and inositol, presumably due to increased intestinal synthesis of thiamine. It thus seems logical to assume that some substance needed by the rat was produced by the intestinal flora when starch was the dietary carbohydrate in our diets or when p-aminobenzoic acid and inositol were administered, but not with sucrose in the absence of p-aminobenzoic acid and inositol.

The data in table 1 show that at the time of appearance of corneal vascularization, the rats on the protein-free diet had undergone a significant reduction in blood hemoglobin and total plasma protein levels. However, the drop of 0.89 gm in hemoglobin level and of 0.54 gm in total plasma protein level is probably not of great practical importance as such. The work of Metcalf, Favonr and Stare ('45), and of Chow, Allison, Cole and Seeley ('45) would indicate that the decreased levels of hemoglobin and plasma protein were accompanied by a considerable decrease in the amount of circulating blood. The decrease in the plasma protein level probably is a result of a decrease in the albumin fraction only (Chow, Allison, Cole and Seeley, '45; Zeldis, Alling, McCoord and Kulka, '45). By the time when the corneal vessels had

regressed, the total plasma protein had returned to a level not significantly different from that of the control rats. The hemoglobin level had risen but was still below that of the control rats.

The rise in hemoglobin level in the control rats during the course of the experiments is in accord with what one might expect in rats of this age, since according to Creskoff, Fitz-Hugh and Farris ('42) newborn rats average 75% hemoglobin (Sahli); then, after birth, there is a slow irregular increase toward the adult level of 101% (100% = 15.6 gm per 100 ml of blood).

SUMMARY

Rats 50 days of age or older, when placed on a diet devoid of protein, develop extreme corneal vascularization. By the time the vascularization appears, blood hemoglobin and total plasma protein show a slight decrease in level. When the rats are returned to an adequate diet, the vessels regress.

ACKNOWLEDGMENT

This study was aided by grants from the John and Mary R. Markle Foundation and from Merck and Co. We wish to thank Dr. Harris Isbell, Dr. Sam Singal, Dr. Philip Dow and Dr. Lane Allen for suggestions and assistance. Elizabeth J. Thompson cared for the animals used and Nell C. Murphey gave technical assistance.

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PLATE 1

EXPLANATION OF FIGURES

1 to 6 Anterior and oblique views of injected corneas from three rats, showing vascularization resulting from protein deprivation. The rats had been on the protein-free diet for 20, 20, and 37 days, respectively. ($\times 15$ reduced approximately a third).

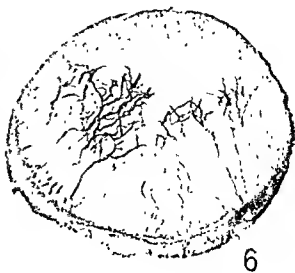
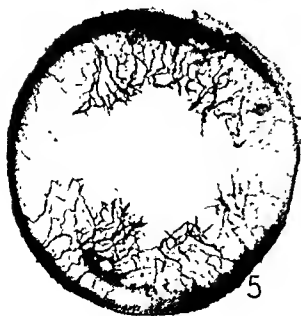
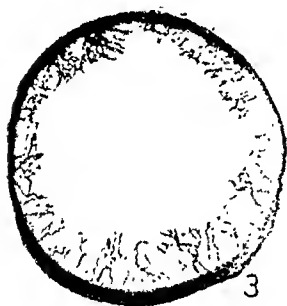
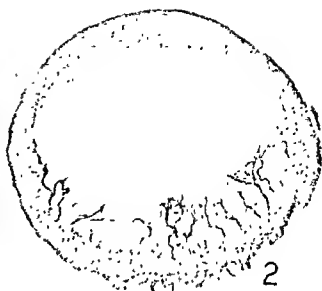
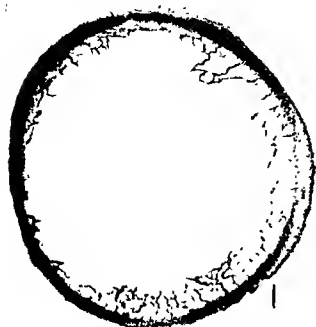


PLATE 2

EXPLANATION OF FIGURES

7 and 8 Anterior and oblique views of an injected cornea from a rat showing vascularization resulting from protein deprivation. The rat had been on the protein-free diet for 37 days.

9 Anterior view of injected cornea shown in figure 10.

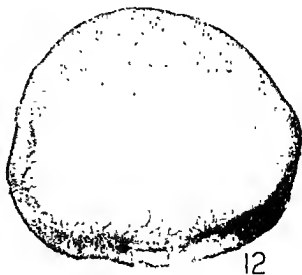
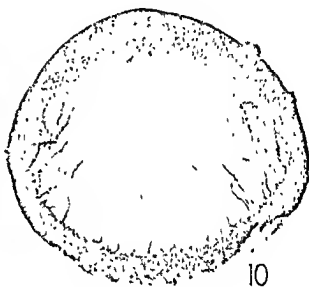
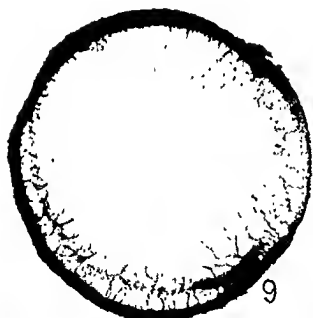
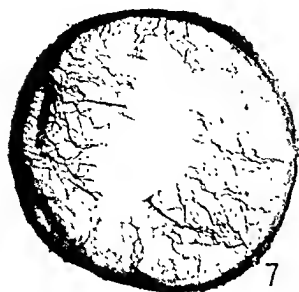
10 to 12 Oblique views of injected corneas from three rats showing regression of vascularization. The cornea in figures 9 and 10 is from a rat which had been on the protein-free diet 36 days followed by 22 days on the control diet.

The corresponding periods for the others rats were:

11 Protein-free diet, 32 days and control diet, 13 days.

12 Protein-free diet, 36 days and control diet, 115 days.

(All $\times 15$ reduced approximately a third.)



DENTAL CARIES IN THE SYRIAN HAMSTER

II. A PRELIMINARY STUDY OF THE EFFECT OF THREE DIFFERENT RATIONS ON CARIES ACTIVITY¹

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TWO FIGURES

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Carious lesions in the molar teeth of Syrian hamsters apparently are associated with many of the fundamental caries-producing mechanisms common to human lesions (Keyes, '46). In previous work a low incidence of caries in hamsters has been associated with appreciable amounts of fluoride in either food or drinking water (Dale, Lazansky and Keyes, '44; Dale and Keyes, '45). In their natural environment hamsters are omnivorous but live largely upon whole grains which they gather in fields and store in burrows. Part of the present study was designed to determine the caries-producing effect of a whole grain diet of low fluoride content. Sugar was incorporated in a known caries-producing ration to determine whether any appreciable difference in caries activity followed its addition to the diet.

Dry yellow corn was selected as a low-fluoride whole grain ration. The Hoppert-Webber-Canniff formula ('32), previously found to be caries-producing was used for purposes of comparison. Sucrose was added to the latter mixture to

¹ This work was made possible, in part, by a grant of the Eastman Dental Dispensary of Rochester, N. Y. Abstract presented before the Twenty-third General Meeting of the International Association for Dental Research, Chicago, Ill., May 27, 1945.

determine whether its incorporation in the diet affected caries activity.

EXPERIMENTAL

All hamsters were obtained from an inbred colony maintained on a stock diet of commercial rabbit food.² From twenty litters between 4 and 5 weeks old, 100 animals were distributed at random into five groups of nineteen to twenty-one animals each. In the three groups used for this study, females ranged from 36 to 72 gm in weight (av. 49 gm); males, from 35-58 gm in weight (av. 48 gm).

Group I animals (12 females and 8 males) were given a basal ration of whole yellow corn. After the sixth day one-half of the group (6 females and 4 males) received supplements of a 90% whole powdered milk, 10% dry powdered alfalfa mixture. From the sixth to thirteenth day this supplement was available *ad libitum*. The allowance was then restricted to 20 gm per animal per week. The fluoride content of the milk-alfalfa mixture was 1.64 ppm.

Group II animals (12 females and 7 males) received the standard Hoppert-Webber-Canniff (H. W. C.) diet: 60% corn meal; 30% whole powdered milk; 6% linseed meal, 3% alfalfa meal, and 1% NaCl. A screened sample of the corn meal was distributed as follows: 100% through a 20-mesh screen; 40% on a 40-mesh screen; 10% on a 60-mesh screen; and 50% through a 60-mesh screen.

Group III animals (12 females and 8 males) were fed a diet containing two parts of the H. W. C. mixture and one part of confectionery sugar.

Rations were analyzed for moisture, protein, fat, ash, and fluoride by official methods of the A. O. A. C. These values are reported in table 1 together with estimations calculated from reliable sources (Sherman, '41; Jacobs, '44; Handbook of Nutrition, '43; and The Canned Food Reference Manual, '43).

Animals were kept in metal cages with wood shavings for bedding. Diets and distilled water were available *ad libitum*.

² Purina Rabbit Checkers.

TABLE 1
Percentage values of some constituents in diets.

RATION	HOPPERT- WEBER- OANNIFF	HOPPERT- WEBER- CANNIFF AND SUGAR	WHOLE CORN	NIBBLED CORN	EDIBLE CORN FRACTION
Moisture	7.6	5.2	10.6	11.1	9.9
Protein	17.4	11.6	10.1	9.4	11.5
Fat	10.8	7.2	4.0	1.4	9.2
Carbohydrate and N.F.E. ¹	56.	71.	73.4	73.9	73.2
Ash	4.6	3.1	1.3	0.5	2.9
Fluoride	0.87 ppm	0.92 ppm	0.61 ppm 0.25 ppm ²	0.16 ppm ²	0.43 ppm ²
Calcium ¹	.313-.321	.208-.214	.015-.029	.016	
Phosphorus ¹	.408-.498	.272-.332	.23-.43	.007	
Vitamin A ¹	10 I.U./gm	6.6 I.U./gm	2-8 I.U./gm	7-7.5 I.U./gm	
Vitamin D ¹			0	0	0
Thiamine ¹	4.2 µg/gm	2.8 µg/gm	3.4 µg/gm	7-3 µg/gm	
Niacin ¹	12 µg/gm	8 µg/gm	2-26 µg/gm	+	
Riboflavin ¹	5.3 µg/gm	3.5 µg/gm	1.4 µg/gm	+	

Fresh alfalfa supplements supplied additional amounts of Ca and P. Vitamins A, E, K, etc

¹Values calculated from Sherman, '41; Jacobs, '44; Handbook of Nutrition '43; and The Canned Food Reference Manual, '43.

²Comparable values in a corn sample obtained after completion of this experiment.

Supplements of fresh green alfalfa were supplied to each group once a week. The animals were maintained on these rations for 100 days after which time they were sacrificed.³ From heads fixed in 10% formalin, the jaws were removed, stripped of soft tissue, and dried. The teeth were examined under a low-power dissecting microscope (1.5 × 10), and all gross carious lesions were charted and scored by a method previously reported (Keyes, '44).

RESULTS

With one exception all animals survived the experimental period. A severe epidemic of "sniffles" passed through the

³One female in Group I died on the fifty-fifth day of the experiment. There was no evidence of caries in the teeth at this time. These data are not included in final results.

entire colony during the sixth and seventh week and retarded growth slightly. Otherwise the animals remained in good health.

All Group I hamsters on whole corn grew poorly, except those which received the milk-alfalfa supplement. Weight curves of hamsters on the H. W. C. formula (Group II) were the best of the three groups. Animals fed the H. W. C. ration mixed with sugar (Group III) grew better than those on whole corn but not so well as those on the standard H. W. C. ration (fig. 1).

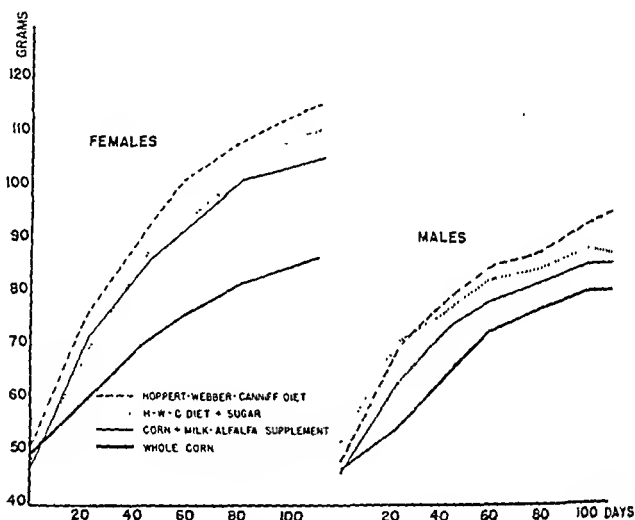


Fig. 1 Growth curves for male and female hamsters.

The incidence of dental caries in the three groups is indicated in tables 2 and 3. The whole corn ration was not conducive to caries activity. Since the milk-alfalfa supplement did not affect the incidence of tooth decay favorably or unfavorably, all caries values for Group I are reported together. The H. W. C. ration, prepared with a semi-fine corn meal, was not high caries producing. A marked increase in caries activity was found in animals that received sugar in their ration. Not only were a greater number of teeth involved, but the number of cavities and extent of cavitation were also increased.

TABLE 2

Caries incidence as found in individual molars.

MOLAR TEETH	GROUP I		GROUP II		GROUP III	
	% molars affected	Ave. % of teeth decayed	% molars affected	Ave. % of teeth decayed	% molars affected	Ave. % of teeth decayed
Female						
<i>Maxillary</i>						
1st	0	0	0	0	62.5	6.1
2nd	0	0	4.3	0.09	75.0	34.8
3rd	0	0	0	0	83.5	45.4
<i>Mandibular</i>						
1st	0	0	0	0	62.5	6.1
2nd	8.4	0.2	33.3	0.4	79.1	17.2
3rd	0	0	0	0	74.1	7.7
Male						
<i>Maxillary</i>						
1st	0	0	0	0	56.2	5.2
2nd	0	0	21.4	2.8	62.5	31.1
3rd	6.2	0.7	71.5	10.0	87.5	59.5
<i>Mandibular</i>						
1st	0	0	7.2	3.6	62.5	20.1
2nd	50.0	3.1	93.0	25.1	68.8	10.7
3rd	0	0	35.7	1.2	68.8	21.7

TABLE 3

Caries incidence in relation to experimental diets—Summary table.

	GROUP I		GROUP II		GROUP III	
	Whole corn		H W C. diet		H W C. sugar	
Number of animals in group	11♀♀	8♂♂	12♀♀	7♂♂	12♀♀	8♂♂
Percentage of animals affected	18	62	58	100	100	100
Percentage of carious teeth	1.5	9.3	6.3	38.1	69.4	67.7
Average number of carious lesions	0.2	1.3	0.7	4.6	10.3	11.1
Average score ¹	0.1	1.8	0.2	19.3	51.1	64.1

¹Of a possible score of 232, representing complete molar crown destruction.

Under the conditions of this experiment, male hamsters showed a tendency to be more susceptible to dental caries than female. This difference was most apparent in Groups I and II. In Group III the difference is less apparent and is suggested only when the over-all average of scores is considered.

DISCUSSION

As a preliminary approach to more detailed studies, this experiment was designed to compare the effect of three different diets on the incidence of dental caries in hamsters. The chemical constituents reported in table 1 are but a partial indication of the total nutrients in the rations. However, from these values alone it would be difficult to predict the effect of these diets on caries activity. There appears to be no definite correlation between chemical constituents and the caries experience of the different groups. The type and activity of the fluoride compounds in the rations is not known, but apparently they were not present in sufficient quantity to exert a caries-inhibiting effect.

Maxillary second and third molars and mandibular second molars showed a high degree of susceptibility, but sufficient data are not available to determine the relative susceptibility of each tooth. No explanation of the sex difference in caries activity can be offered at present. Arnold ('42) found more lesions in male than in female animals but did not conclude that a sex difference existed. Dale and Keyes ('45) also report such findings.

Hamsters eating the dried corn nibbled out the germ and consumed only small amounts of the hard endosperm. The slow growth of animals on this ration suggested nutritional deficiencies.⁴ Despite the possible inadequacies of this ration, animals on this diet had as little evidence of caries activity as we have observed. To conclude that the low incidence of dental caries in this group is due to an optimum or even adequate diet is difficult. It is evident, however, that neither

⁴ There has been complete growth failure in a small group of hamsters subsequently maintained on a whole corn ration without supplementation of any kind.

the physical nature of the cereal particles comminuted by the animals nor the chemical composition of the kernel fraction consumed was conducive to the processes that initiate carious lesions.

Weight curves are used as acceptable criteria of animal progress on a given regime. In dietary studies poor growth generally indicates the lack of sufficient food or the absence of essential nutrients. Since rations were available *ad libitum*, the poor growth in Group I was undoubtedly due to the scarcity of essential nutritional constituents. The suggestion is sometimes advanced that a high incidence of caries is the result of dietary inadequacy and lack of protective foods. On this basis one would expect to find high caries scores in animals which grew the poorest and low scores in those which grew the best. The opposite was found in this study. The possibility has been suggested that the greater incidence of caries in males may indicate that the rations did not contain the most desirable concentration of nutrients needed by the hamster, and therefore, male animals, which presumably grow more and have a greater demand for essential nutrients, were more susceptible to tooth decay. If this were the case, one would expect to find the highest incidence of caries in males on whole corn and the lowest incidence of caries in males whose weight curves exceeded females, e.g., males on the H. W. C. and H. W. C.-sugar rations compared with females on whole corn. However, male animals with poor growth had very little evidence of caries and those whose growth exceeded the corn-fed females had 200 to 600 times more tooth substance destroyed. This presumption is untenable, however, because male hamsters normally weigh less than the females.

That inadequate diets may not be associated with dental caries has been reported for human beings. A low incidence of dental caries has been observed in people suffering from severe rickets, osteomalacia, and other nutrition deficiencies (Taylor and Day, '39; Day, '44; Staz, '38).

Although the H. W. C. ration has been adequate for rats (Hoppert, Webber and Canniff, '32), the complete adequacy of this diet for hamsters has not been tested. Animals receiving this ration have remained in good health and shown no symptoms of nutritional deficiency (Dale, Lazansky and Keyes, '44; Dale and Keyes, '45). Since typical rickets can be produced in hamsters only on low phosphorus and vitamin D deficient rations (Jones, '45), this diet cannot be considered rachitogenic, especially when supplemented with fresh greens. In contradistinction to rats, however, caries activity is not associated with cusp fractures from coarse corn meal in the diet and actually increases on finer corn meal. In part, this variation may be due to differences in crown morphology and type of occlusion between the two species.

In this experiment the caries experience of animals on the H. W. C. ration was considerably lower than expected from previous work. A factor influencing the caries-producing properties of this diet may have been the semi-fine grind of the corn.⁵ The particle size of one-half the corn meal was too large to collect in the sulci and fossae of the teeth (fig. 2). Such particles may have had a detergent action.

The effect of sugar on experimental caries in rats has been confusing. After extensive survey of the literature on rat caries, Cox ('44) concluded that sugar will promote the enlargement of cavities but fails to cause the initial appearance. However, Shaw and coworkers ('44) and Schweigert et al. ('45) report a high incidence of caries activity in cotton rats fed high percentages of sugars in diets. Recently, McClure ('45) reported that white rats developed a significant incidence of microscopic caries when fed synthetic diets containing excessive quantities of sucrose and glucose.

In hamsters the addition of powdered sugar greatly increased caries activity and affected not only the incidence of

⁵ The corn meal used in previous studies (Dale et al., '44; '45) sifted as follows: 24% on a 40-mesh screen, 13% on a 60-mesh screen, and 63% through a 60-mesh screen.

lesious but also the size. Whether the sugar per se was responsible for this increase in caries activity or whether its combination with other constituents in the ration formed a caries-producing combination is not known. The data indicate that the mixture of sucrose into the diet was conducive to initiation of lesions as well as development.



Fig. 2 Three maxillary molars in situ compared with corn meal fractions in the Hoppert-Webber-Canniff diet: A. corn fraction passing 60-mesh screen, 50%; B. corn fraction retained by 60-mesh screen, 10%; C. corn particles retained by 40-mesh screen, 40%.

CONCLUSIONS

Data from this experiment suggest that, in hamsters, caries activity is related more to the dietary constituents than to the nutritional value of ingredients. The physical character of the ration and the sex of the animal apparently influence caries activity. Emphasis is placed on the observation that there may be no correlation between growth curves and caries activity. Inadequate rations are not necessarily caries-producing, and rations which support satisfactory growth may have high caries-producing value.

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THE GROWTH AND MAINTENANCE UTILIZATION OF DIETARY PROTEIN¹

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FIVE FIGURES

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The biological value of protein has been defined as the fraction of the absorbed protein nitrogen that is retained in the body (Thomas, '09). This expression of the nutritive quality of proteins as it has been modified by Mitchell ('24) may be applied to either growing or adult animals. However, in the adult the retained nitrogen is utilized only for the maintenance of the nitrogenous integrity of the tissues while in the growing animal increases in body protein as well as maintenance requirements account for the retained nitrogen. The purpose of this investigation was to measure the maintenance and growth utilization of several proteins in young rats and to study the influence of the amount of protein ingested.

EXPERIMENTAL

Weanling male albino rats³ were individually caged in a room with temperature controlled at 78°F. and were fed a commercial diet⁴ for 3 days. This pre-test period was included so as to provide time for some readjustment of the

¹ Parts of this paper were reported at the American Chemical Society meeting, New York, 1944, and the New York Academy of Sciences, New York, 1945.

² Present address: Sharp and Dohme, Inc., Glenolden, Pennsylvania.

³ Sprague-Dawley.

⁴ Purina Fox Chow.

TABLE 1

Composition of the basal diet.

Protein sources that were incorporated in the diet replaced carbohydrate and fat so that the respective combinations were isocaloric in nature.

BASAL MIXTURE		VITAMIN MIXTURE ¹ PER 100 GM OF BASAL MIXTURE	
Sucrose: varied inversely with protein added		Riboflavin	400
Fat ² 15%		Thiamine	400
		Pyridoxine HCl	400
Salt mixture ³ 4%		Niacin	2,500
Fiber ⁴ 2%		Calcium pantothenate	1,100
Vitamins A and D ⁵ +		Para amino benzoic acid	7,500
Vitamin mixture ¹ +		Choline HCl	200,000
		Inositol	20,000
		2-Methyl 1,4 naphthoquinone	50
		alpha Tocopherol	2,500

¹ The tocopherol was added to the fat and the water-soluble components were incorporated in the sucrose.

² Soybean oil was added to make a total of 15%.

³ Wesson ('32) modification of the Osborne-Mendel salt mixture with a trace of ZnSO₄ added.

⁴ Cellu flour, from Chicago Dietetic Supply Co.

⁵ Cod liver oil was administered by mouth with an eyedropper twice weekly.

animals to their new environment. The rats were then divided according to weight into twenty-eight groups of eight rats each so that the average body weight of each group was approximately 60 gm. Four crude protein sources⁵ representing a wide range in nutritive quality were incorporated into the basal diet described in table 1, so that seven different

⁵ Spray-dried whole eggs extracted three times with cold petroleum ether. The protein content ($N \times 6.25$) was 76%.

Soyflour (no. 1) kindly supplied by Dr. J. Hayward, Archer-Daniels-Midland Co., Minneapolis, Minn. The analysis was protein ($N \times 6.25$) 48.9% and fat 0.3%. The product was commercially processed so as to develop a high nutritive quality protein.

Soyflour (no. 2) kindly supplied by Dr. J. Hayward, Archer-Daniels-Midland Co., Minneapolis, Minn. The analysis was protein ($N \times 6.25$) 49.3% and fat 0.3%. The product was commercially processed so as to develop a protein of intermediate nutritive quality.

Wheat gluten purchased from the Huron Milling Co., Harbor Beach, Michigan. The analysis was protein ($N \times 6.25$) 75%.

levels of each protein could be fed. Each diet was checked for protein content by Kjeldahl nitrogen determinations. For 42 days the rats ate these diets ad libitum and careful observations of body weight and food consumption were made. Feces were collected over 4-day periods during the second, fourth, and sixth weeks and apparent digestibility was calculated from the difference between the total intake and fecal output of nitrogen. An average of the three fecal collection periods was used to determine the total protein apparently absorbed during the 42-day period.

At the conclusion of the test period the rats were killed with ether and after washing out the contents of the stomach and large intestine the carcasses were digested with sulfuric acid and Kjeldahl nitrogen was determined. Carcass nitrogen was also measured on a group of rats at the start of the experiment so that calculation of gains in carcass protein during the 42-day experimental period could be made.

RESULTS

Determination of body protein gain

With increased amounts of the four crude protein sources in the diet there was an increase in protein consumption (see table 2). The increased consumption of protein enhanced the growth rate until a maximum was obtained. The maximum body protein gain was essentially the same for three of the crude proteins, but the poorly heated soyflour (soyflour no. 2) supported a maximal protein gain that was distinctly below the others (see fig. 1). The anomalous result may be related to the peculiar influence of heat upon the nutritive quality of soybean protein. With increasing consumption of dried egg protein it would appear that body protein gain reached a maximum and then decreased slightly. Decreased growth and body protein gain at high levels of protein intake have been observed in other studies (MacKay et al., '41; Bosshardt et al., '46).

TABLE 2

Growth and maintenance data derived from studies on 4 crude protein sources. Values are expressed as averages per rat for 42 days.¹

1	2	3	4	5	6	7	8
GROUP NO.	PROTEIN IN DIET	AVERAGE BODY SURFACE AREA	PROTEIN CONSUMED	PROTEIN APPARENTLY ABSORBED	BODY PROTEIN GAINED	PROTEIN FOR MAINTENANCE (EGG)	PROTEIN FOR ENERGY ALONE
	%	cm ²	gm	gm	gm	gm	gm
Whole egg							
1	4.1	182	7.4	6.3	1.5	5.3	-0.5
2	5.8	220	16.9	15.0	9.1	6.4	-0.5
3	7.9	247	25.9	23.6	16.0	7.1	0.5
4	9.9	286	39.8	35.8	27.2	8.3	0.3
5	13.7	297	57.6	51.8	29.7	8.6	13.5
6	19.0	297	77.4	70.5	30.7	8.6	31.2
7	38.7	303	153.0	142.0	29.0	8.8	104.0
Soyflour no. 1							
1	6.0	188	14.7	12.5	3.9	5.5	3.1
2	7.7	204	23.8	20.2	6.5	5.9	7.8
3	9.4	227	31.8	27.3	11.7	6.6	9.0
4	11.5	242	41.0	35.7	15.1	7.0	13.6
5	14.8	262	59.5	51.7	20.5	7.6	23.6
6	18.5	280	79.6	69.3	25.6	8.1	35.6
7	27.7	286	117.0	100.0	27.9	8.3	63.8
Soyflour no. 2							
1	6.1	164	12.4	9.7	3.4	4.7	1.6
2	8.5	172	19.3	15.8	5.5	5.0	5.3
3	9.7	185	24.0	19.7	7.5	5.3	6.9
4	12.6	207	37.5	31.6	12.6	6.0	13.0
5	14.5	234	55.6	46.3	18.0	6.8	21.5
6	16.9	232	58.8	49.4	19.5	6.7	23.2
7	31.1	230	98.0	82.3	19.3	6.6	56.4
Wheat gluten							
1	8.4	173	20.2	19.2	1.1	5.0	13.1
2	10.2	178	21.6	20.5	2.0	5.1	13.4
3	12.4	181	27.1	25.8	2.6	5.2	18.0
4	14.6	188	33.4	31.7	3.8	5.4	22.5
5	20.8	211	56.8	54.0	7.4	6.1	40.5
6	31.5	242	106.0	102.0	15.2	7.0	80.0
7	43.0	287	187.0	179.0	27.5	8.4	143.0

¹ Column 1 — Eight rats in each group.

Column 2 — Protein ($N \times 6.25$) determined by Kjeldahl analysis of each diet.

Column 3 — Average body surface area during the 42-day experimental period. Each figure is also the average of the group of eight rats.

Column 4 — Ad libitum food intake corrected for spilled food and protein intake calculated from analysis of the diets.

Column 5 — Protein apparently absorbed calculated according to the equation: protein intake minus fecal protein = protein apparently absorbed.

Column 6 — Body protein gained determined from carcass nitrogen at the end of the experiment minus carcass nitrogen of control group taken at the start of the experiment.

Column 7 — This calculation is based on the observation that maintenance requirements for protein are proportional to body surface area. Furthermore, it is assumed that whole egg protein at low levels in the diet is entirely used for maintenance. Therefore, at any given body size the amount of protein used for maintenance alone is equal to the mg of egg protein ($N \times 6.25$) that was found necessary for the maintenance of nitrogen equilibrium (average of two values given in table 3) multiplied by the body surface area in square decimeters. The entire calculation of protein used for maintenance during the experimental period is: (mg egg N per 100 sq. cm. B.S. per day) \times (protein conversion factor) \times (days of experimental period) \times (average body surface area during experimental period in square decimeters). An example of this calculation using group no. 1 receiving soyflour no. 1 (table 2) is: $11.1 \times 6.25 \times 42 \times 1.88 = 5500$ mg protein.

Column 8 — It is assumed that protein neither used in building new tissue nor maintaining the integrity of tissues is utilized directly as a source of energy. The calculation in this column represents the difference between column 5 and the sum of columns 6 and 7.

Calculation of the maintenance utilization of protein

The second major use of dietary protein is in the maintenance of the nitrogenous integrity of the tissues. This type of protein utilization may be defined as the minimum amount of absorbed protein that will sustain exact nitrogen equilibrium; in other words maintain the animal without overall gain or loss of body protein. The body protein gains that are described in figure 1 may be used to calculate the minimum

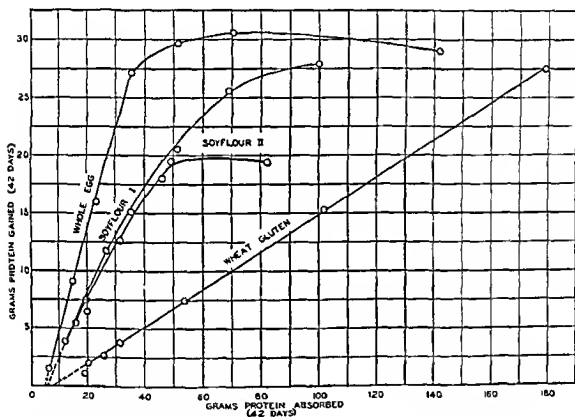


Fig. 1 Gain in body protein following the ad libitum ingestion of rations containing varying amounts of protein.

maintenance requirements of young, 60-gm rats, by extrapolating the curves to the point of zero body protein gain. This is in essence the method used by Osborne and Mendel ('15) for the measurement of the maintenance requirement. It has been shown by others (Olson and Palmer, '40; Smuts, '35) that the so-called endogenous urinary nitrogen excretion varies directly with body size. Furthermore, the best correlation appears to be with body surface area. It was considered

possible that the maintenance requirements established by extrapolation may have been influenced by reduced caloric intake due to the lowered protein content of some of the diets. Also it was possible that these maintenance requirements would not remain a constant function of surface area over the wide range of body size from immature to adult rats. Therefore, four groups of adult rats of the same sex and strain as used in the above study were fed diets containing the four crude protein sources at low levels that would either maintain the rats in approximate nitrogen equilibrium or would produce a slightly negative nitrogen balance. By plotting nitrogen balance against nitrogen apparently absorbed it was possible to interpolate to the point of exact equilibrium. The general principle of this method of establishing maintenance requirements of adult rats was the same as that described by Melnick and Cowgill ('37) in their studies on dogs. A comparison of the protein requirements for maintenance of nitrogen equilibrium as measured by extrapolation of protein gain in young rats and the conventional nitrogen balance in adults is presented in table 3 (first section).

TABLE 3

Comparison of protein requirements for the maintenance of nitrogen equilibrium as measured by two different methods involving the adult and the young rat (columns 2 and 3). Columns 4 and 5 give a comparison of the nutritive values of four protein sources for maintenance of nitrogen equilibrium and for growth.¹

PROTEIN SOURCE	NITROGEN BALANCE (ADULT)	CARCASS NITROGEN (YOUNG)	MAINTENANCE (ADULT)	GROWTH (YOUNG)
1	2	3	4	5
<i>mg N/100 sq. cm²</i>				
Whole egg	11.2	11.0	100	100
Soyflour no. 1	13.6	13.2	83	55
Soyflour no. 2	15.1	15.3	73	45
Wheat gluten	18.0	17.6	62	21

¹ Relative maintenance values are calculated from data on the minimum nitrogen intake necessary for the maintenance of nitrogen equilibrium (columns 2 and 3) with whole egg protein arbitrarily set at 100. Relative growth values are calculated from the maximal protein efficiency ratios (Barnes et al., '45) with whole egg protein arbitrarily set at 100.

² Body surface area estimated from the formula Surface Area = $11.36 \times \text{Body Weight}^{\frac{2}{3}}$.

When expressed as a function of surface area the results are essentially the same for both methods. The curves of body protein gains for the two soyflours that are presented in figure 1 show that the extrapolation comes to exactly the same point for both protein sources. The rats receiving the soyflour no. 2 diets were started at body weights that were approximately 10 gm lighter than the others. When expressed as a function of surface area there was a definite difference in the maintenance requirements for the two proteins.

Graphic representation of protein utilization

The results of the above study substantiate the conclusions of others that maintenance requirements are directly proportional to body surface area. Furthermore they provide a basis for the calculations of the amount of protein that was utilized for maintenance of the nitrogenous integrity of the tissues during the 42-day growth period that was used in this study. The average body surface area of each group of rats during the 42-day experimental period was calculated from the growth curves.⁶ The assumption was made that whole egg protein is 100% utilized for maintenance at low levels of intake and the average amount of protein utilized for maintenance of each of the experimental groups was established on this basis. An illustration of this calculation is presented in a footnote to table 2. The assumption that whole egg protein is utilized as a perfect protein for maintenance may be open to criticism but the work of Mitchell and Carman ('26), Murlin et al. ('38) and others indicates that the utilization of this protein mixture approximates 100%.

With this assumption it was possible to express the maintenance and the growth utilization of protein as a percentage of the protein apparently absorbed. Also the difference between the sum of these two types of utilization and the total absorbed protein must represent protein that was directly

⁶ Growth curves for the rats used in these studies have been presented elsewhere (Barnes et al., '45).

utilized for energy. The data that are pertinent to these calculations are given in table 2 and the graphic representation of the three types of utilization for the four crude protein sources is given in figures 2, 3, 4 and 5. The expression "waste energy" has been used to designate the protein that was not used for growth or maintenance. The term "waste" is intended only to indicate a type of utilization that is not unique to proteins.

According to concepts developed by Mitchell the biological value of a protein is the sum of the nitrogen utilized for maintenance and growth expressed as a percentage of the absorbed nitrogen, the latter being corrected for metabolic fecal nitrogen. Absorption data presented here have not been corrected for metabolic fecal nitrogen but it is probable that the qualitative relationships that will be dealt with are not affected by this omission. This value which is the complement of "waste energy" also has been represented graphically in figures 2, 3, 4 and 5. In each case the curves have been extrapolated to the theoretical level of protein absorbed where there would be zero body protein gained. In the case of egg protein the maintenance at this point was assumed to account for 100% of the absorbed protein. In the other examples the percentage of absorbed protein utilized for maintenance at this level of protein intake was calculated from the maintenance data that were presented earlier. In each case the biological value is entirely accounted for by the maintenance value. According to Allison and Anderson ('45) the biological value should remain at this same level through the range of negative nitrogen balance to the point of zero protein intake.

At levels of protein intake in excess of the amount required for maintenance alone there is a sharp increase in the percentage utilization for body protein gain until a maximum is reached. This maximum is clearly defined for all proteins except wheat gluten. In this case body protein gain appears to establish a plateau. Percentage utilization for maintenance

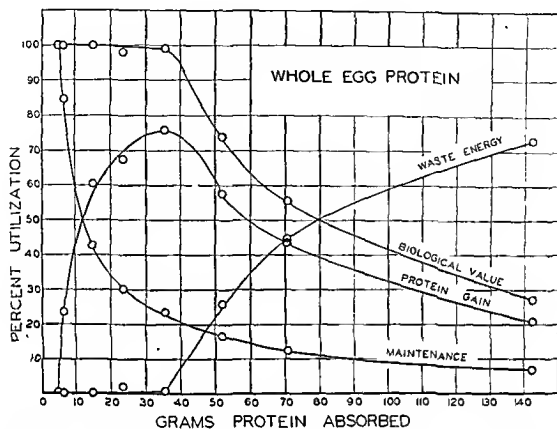


Fig. 2 The comparative utilization of whole egg protein for maintenance and growth.

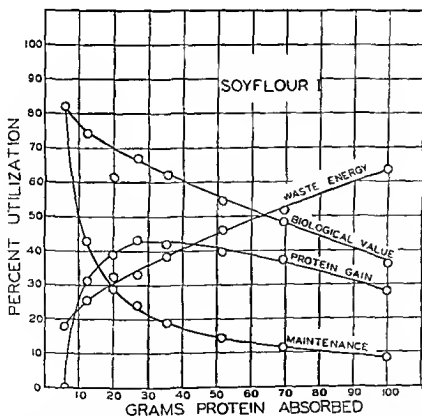


Fig. 3 The comparative utilization of a well heat-treated soyflour protein for maintenance and growth.

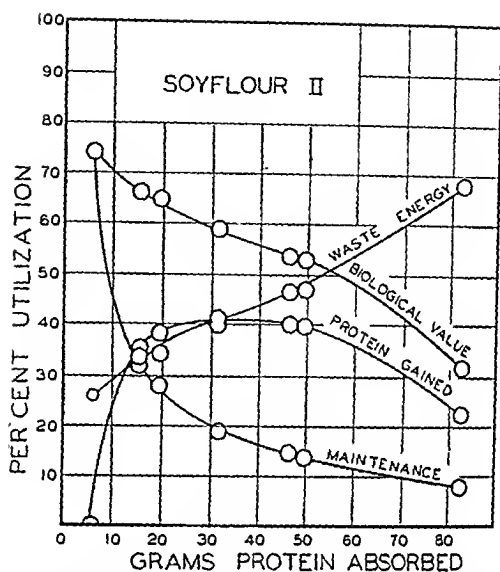


Fig. 4 The comparative utilization of a poorly heat-treated soyflour protein for maintenance and growth.

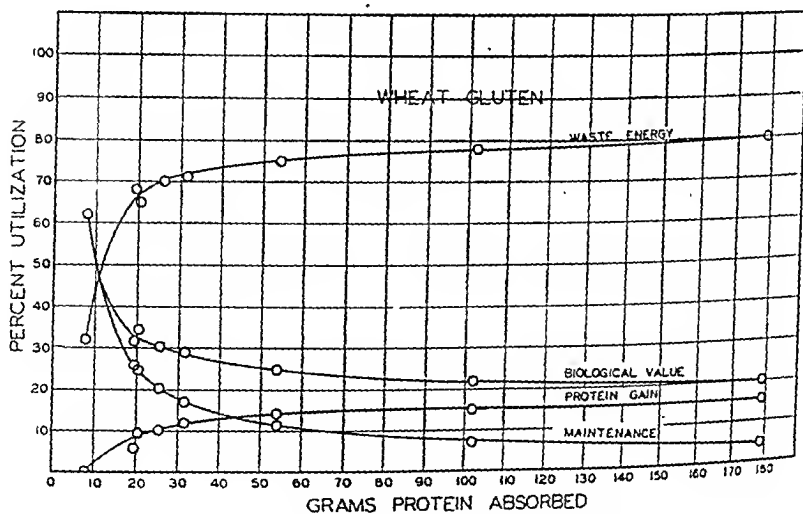


Fig. 5 The comparative utilization of wheat gluten protein for maintenance and growth.

declines sharply with increasing protein consumption and approaches an asymptote at the higher levels of intake. The resultant biological value exhibits an immediate decline when protein intake is increased except in the case of egg protein. Here the biological value remains in the proximity of 100 until maximal growth utilization is attained. Following this comes the characteristic decline. The observation that this protein source has a fairly constant biological value of approximately 100 over a wide range of protein intake lends support to the original assumption that it was completely utilized for maintenance at a very low level of intake.

DISCUSSION

The observation that the biological value of proteins decreases when the level of intake is increased substantiates Mitchell's ('24a) conclusions. The graphical representation of changes in biological value and in component utilizations that make up the biological value offers a more thorough understanding of the reasons for the change.

There is a difference in the quantitative protein requirements for growth and maintenance. This is a well known observation and the results presented here provide further confirmation. If the nutritive value of whole egg protein for both growth and maintenance is arbitrarily set at 100 and the relative values for the three remaining protein sources are calculated, the relationships given in table 3 (second section) are obtained. It will be seen that the peak utilization of the poorest protein source, wheat gluten, for maintenance is approximately 62% while the maximal utilization for growth is only 21%. These data are too incomplete to be used in drawing detailed conclusions regarding the relationship between requirements for growth and maintenance, but they do show the marked difference in the amounts required for these two types of utilization.

If the biological value of a protein is measured in growing animals and is, therefore, composed of a growth as well as maintenance utilization, the relative participation of these

two factors will vary, depending upon the amount and the quality of the protein that is fed. The changes in growth and maintenance utilization with increasing consumption of protein is readily seen in figures 2, 3, 4 and 5. Keeping in mind the differences in protein requirements for growth and maintenance this demonstrates that the significance of the biological value as well as the actual numerical value that is obtained is dependent upon the level of protein-intake. The change in the relative participation of growth and maintenance to the biological value with different quality proteins is shown in table 4. With egg protein approximately 77% of the biological value is due to growth and 23% to maintenance. With

TABLE 4

Relative participation of growth and maintenance in the establishment of the biological value when approximately 10% protein is included in the diet.

PROTEIN SOURCE	PROTEIN APPARENTLY ABSORBED PER RAT/42 DAYS	BIOLOGICAL VALUE	RELATIVE PARTICIPATION OF GROWTH	RELATIVE PARTICIPATION OF MAINTENANCE
	gm	%	%	%
Whole egg	36	99	77	23
Soyflour no. 1	27	87	65	35
Soyflour no. 2	20	64	59	41
Wheat gluten	20	35	26	74

¹ Calculated from data in either column 6 or 7 of table 2 at the level of each protein in the diet that is nearest to 10%.

the lower quality proteins this ratio decreases so that with wheat gluten only 26% of the biological value is due to growth while 74% is due to maintenance. These data are for rats that are receiving a diet composed of approximately 10% protein. Equalization of the protein intake may reduce these differences slightly, but will not abolish them. These results indicate that the significance of the biological value is not the same for proteins of different nutritive qualities. The conclusion that is drawn from these studies is that the measurement of the combined nutritive quality of dietary proteins for growth and maintenance may result in a numerical evaluation having an uncertain significance. The interpretation of values

would be more definite if growth and maintenance utilization of proteins were determined separately.

In the above calculations of growth and maintenance utilization of dietary protein it has been recognized that these two metabolic processes cannot be clearly separated. However, the existence of the two types of protein utilization has been clearly demonstrated and it would appear that there is sufficient evidence to warrant the conclusion that these two metabolic processes operate simultaneously in the growing animal.

The absolute values for the maintenance utilization of dietary protein that are presented here are derived by calculation and include the assumption that at low levels of intake, egg protein is completely utilized for maintenance. Protein utilization is influenced by a great variety of conditions and part of the values presented here have been derived by indirect calculation. Therefore, stress has been put on the qualitative relationships that were observed rather than implying that the absolute maintenance utilization and the biological values were calculated with extreme accuracy. In spite of the indirect approach that was used it is interesting that the biological values for the four test proteins compare favorably with values that are to be found in the literature.

SUMMARY

1. A study has been made of the growth and maintenance utilization of four crude protein sources covering a wide range of nutritive qualities.

2. When the level of protein intake is increased there is a decline in the fraction of absorbed protein that is utilized for maintenance. At the same time the fraction utilized for growth rises to a maximum and then declines. The net result of these changes is a fall in the biological value.

3. The relative participation of growth and maintenance in making up the biological value varies markedly depending upon the quantity and the nutritive quality of protein that is ingested.

4. Since the protein requirements for growth and maintenance are different the conclusion is drawn that these two factors should be measured independently and not in combination.

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OBSERVATIONS ON PELLAGRA IN AMERICAN PRISONERS OF WAR IN THE PHILIPPINES

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The surrender of Bataan and Corregidor in April and May of 1942 brought approximately 17,000 Americans under the control of Japanese Forces. During the subsequent 3 years of confinement in Japanese prison camps in the Philippines deficiency diseases developed in great numbers.

For 3 months prior to capture, the American Forces were on a diet restricted to about one-half the usual field ration. It consisted principally of polished rice plus small amounts of flour, sugar, and canned foods. Calorie values often fell to less than 1000 cal. per day and there was a marked deficiency in fresh meat, fruits and vegetables.

Malaria was rampant among the troops, and cases of bacillary and amebic dysentery appeared in increasing numbers. The fever and digestive disturbances associated with these diseases contributed to deficiency states.

Toward the end of the 5-month campaign symptoms such as night blindness and nutritional edema became common and occasional cases of mild peroneal paralysis with foot drop appeared. But symptoms of pellagra were not seen.

After the surrender, the Americans received from the Japanese during the summer and fall of 1942 a ration consisting

¹The authors were serving as medical officers with U.S. troops on Bataan and were captured and held by the Japanese from April 9, 1942 to January 30, 1945. The observations included in this paper were made during that time.

essentially of the following: polished rice, 300 gm; vegetables (greens, leaves and vines), 100 gm; flour, 30 gm; sugar, 10 gm; and oil, 10 gm. Approximately once a week an average of 10 gm of fresh meat per man was issued. Thus the diet afforded less than 1500 cal. and it was hopelessly deficient in proteins and vitamins.

Nutritional edema became universal in the first few weeks of prison camp existence varying in degree from mild pedal edema to generalized anasarca. Beri-beri appeared by July, 1942, and it remained always the most severe and widespread deficiency entity.

Symptoms of pellagra first appeared with dramatic suddenness in August, 1942. Within a week 500 men out of a camp of 5000 presented signs and symptoms of the disease. They complained chiefly of soreness and burning of their mouths and tongues aggravated by hot or seasoned foods.

The tip of the tongue presented the typical raw, spotted appearance due to swollen, inflamed papillae. The inflammatory lesion progressed, extending along the margins of the tongue, along the median sulcus and to the under surface. Later ulcerations appeared on the tongue. The angles of the mouth became macerated and presented a perleche-like membrane. A seborrheic lesion appeared about the alae of the nose and spread over the lips and chin. The posterior aspect of the ears was similarly involved in some cases.

The skin lesions seen were similar to but more extensive than those usually described as pellagrous. They occurred on parts of the body exposed to sunlight and they were aggravated by sunlight. An erythematous dermatitis appeared on the dorsum of the hands, on the forehead, and in a butterfly pattern over the cheeks and nose. A similar lesion developed on the prominences of the back, chest and abdomen, on the dorsal aspect of the arms and feet, on the anterior thighs, and on the calves of the legs when these areas were exposed. Protection from sunlight by clothing or the straps of wooden clogs or sandals left a pattern of relatively uninvolved skin sharply demarcated from the pellagrous lesion.

In the more severe cases the mucous membrane of the entire mouth and pharynx became inflamed and edematous. There was a blotchy, reddish-purple, granular appearance to the palate and pharynx. Patches of grayish pseudomembrane were often present. The tongue was swollen, raw and fiery red having a beefy appearance. It was often crenated from pressure against the teeth. Speaking, chewing and swallowing were painful and difficult.

In the areas of dermatitis the subcutaneous tissue became soggy with edema. Vesicles and bullae appeared. Often the bullae became hemorrhagic. Desquamation, rupture of the bullae, weeping eczematous lesions, and secondary infection with crusting, purulent lesions marked the progression of the disease. Other areas of the skin, principally the flexor folds of the knees and elbows, assumed a moderately edematous, blotchy, ecchymotic appearance. We were unable to demonstrate a significant increase in capillary fragility by the tourniquet test.

Skin integrity was so impaired that the slightest trauma would result in a break in the integument with infection and ulcer formation. We believed that nicotinic acid deficiency was an important factor in the etiology of the so called "tropical ulcers" which were seen in great numbers among the prisoners.

Dermatitis of the scrotum was an interesting condition observed as a prodromal as well as a concomitant symptom of pellagra. Men came to sick call complaining of a burning, itching scrotum. Examination revealed an inflamed, edematous, weeping dermatitis of the scrotum which would dry and desquamate or become secondarily infected and exude a sanguino-purulent fluid. The usual signs of pellagra might not appear for as long as 3 weeks later.

The fact that the scrotal dermatitis was alleviated, at least in part, by protection of the scrotum from the friction of clothing again demonstrated the decreased resistance of the skin to trauma. A similar dermatitis occurred in a perianal distribution.

In cases less severe or improving the erythema was followed by thickening, roughening and scaling of the skin which frequently cracked and became infected. There was an associated pigmentation similar to sun tan. During remissions the skin often had a glossy, atrophic appearance.

Many patients exhibiting mild clinical signs of pellagra or giving a previous history of pellagra suffered from digestive disturbances. They complained of postprandial epigastric discomfort, flatulence, pyrosis, foul eructations described as "yeasty" or like rotten eggs, and recurrent attacks of diarrhea. In the presence of so many cases of infections diarrhea and dysentery it was difficult to decide in individual cases of pellagra whether the diarrhea was on an infectious basis or whether it was a symptom of the pellagra. It was believed that the deficiency resulted in inflammation and irritability of the gastrointestinal tract with impaired digestive efficiency and diarrhea and with an increased susceptibility to infection. Diarrhea, whether on a functional or infectious basis, resulted in aggravation of deficiency symptoms.

Later when scant laboratory facilities became available, it was demonstrated that many pellagrins had a reduction or absence of free hydrochloric acid. The impression was that this impairment of gastric secretion was part of the pellagra syndrome. Dilute hydrochloric acid, when available, definitely alleviated these symptoms. The decrease in gastric acidity was possibly a factor in the high incidence of intestinal infestation encountered.

Cases of idiopathic urinary frequency and dysuria with occasional hematuria were common and may have been due to a bladder lesion associated with a generalized involvement of all mucous membranes.

Neurological lesions were common in pellagrous patients, but to what extent these were attributable to thiamine deficiency it is difficult to say. Sensory changes consisted of numbness and tingling, decreased sensation to light and deep touch, and loss of position sense and sensory ataxia. Trophic changes often persisted after subsidence of the dermatitis.

Some showed paresis, foot drop and impairment of deep reflexes while others showed spasticity and paresis and exaggerated deep reflexes. These changes occurred for the most part in the lower extremities.

Symptoms of irritability, moodiness, loss of memory and inability to concentrate which were observed might be ascribed either to deficiency or to psychic trauma from privation, abuse, and crowded confinement. A few patients progressed to disorientation and delirium.

These patients were suffering from a mixed deficiency and it is difficult to estimate how much of the neurological lesions was attributable to nicotinic acid deficiency and how much to thiamine deficiency. Symptoms of peripheral nerve lesions responded to thiamine therapy and symptoms of central nervous system lesions responded to nicotinic acid therapy. In addition, it was our impression that there was considerable overlapping of effects.

Pellagrins exhibited decreased resistance and often succumbed to malaria and dysentery, but no deaths were thought to be due to pellagra primarily.

Although the clinical signs of the disease were slow in manifesting themselves (4 to 8 months) the course of the disease once established was relatively rapid. At Cabanatuan, the symptoms appeared about mid August and by mid to late September some men were exhibiting the more serious lesions. Eventually, the greater part of the command suffered from symptoms to some degree.

Individual susceptibility to dietary deficiency was marked. Men of Spanish and Italian extraction, which races characteristically live on a high carbohydrate diet, were less affected, possibly because of some innate or hereditary quality. Too, the individuals of lighter complexion were more susceptible to the pellagrous lesions, particularly those of the skin. As groups, the larger men and the men doing hard physical work were more susceptible to vitamin deficiency. The men seemed to make a metabolic adjustment to the type of diet and fared better in later months. Actually this may have been due to

the survival only of those physiologically and psychologically capable of living on the diet.

Again it must be emphasized that practically all prisoners exhibited some evidence of B complex deficiency. It was more severe in those who suffered the ravages of dysentery and recurrent malaria and in those who were unable to get even small quantities of food from outside sources. Anyone who could not supplement the Japanese ration of rice and greens soon fell prey to severe pellagra.

The Japanese medical officer showed curiosity and interest in our problem but our request for relief in the form of a more adequate diet as well as specific medication met with little sympathy.

We were given a few bottles of Japanese compressed yeast tablets and we were able to obtain a few bottles of brewer's yeast from Filipino sources. Response to this medication was quite rapid even in amounts as little as 2.0 to 4.0 gm per day. Unfortunately, our supply was utterly inadequate to cope with the great number of cases which developed. Consequently, a yeast culture was prepared using a thin liquid medium composed of cornstarch, sugar, rice and water, seeded with brewer's yeast and wild yeast and allowed to grow for 3 to 5 days.

The number of patients far exceeded our supply of yeast culture, which was limited by lack of materials for the vats as well as for the medium. We were able to treat only the severe cases and those only until a remission with alleviation of the more advanced lesions was obtained. The dosage of the yeast culture varied from 100 to 250 ml depending upon the number of patients being treated. At all times we attempted to obtain and set aside a small amount of extra food for the more serious cases. Surprising improvement followed daily administration of the yeast culture alone or with the addition to the diet of a banana, a handful of peanuts, or part of a cocoanut when these were available. Most cases showed immediate retardation of the disease process with some improvement in 2 to 3 days and marked improvement in 7 to 14

days. Protection from sunlight was an important factor in the healing of the dermatitis.

In December, 1942, American Red Cross supplies of food and medicine arrived in the prison camp. We received enough canned and preserved food to allow an auxiliary daily ration for a period of 2 months of approximately 30 gm of canned meat, 100 gm of canned vegetables, and 20 gm of dried fruit. At about this time the Japanese began almost daily issues of 25 to 50 gm of fresh carabao meat per man and they increased the rice issue to about 500 gm per day. The ration now amounted to about 2000 cal. with 35 gm of protein of which about 10 gm was meat protein. During these months the pellagrins improved rapidly and markedly. The symptoms completely disappeared in most of the patients only to reappear in a milder chronic form in the lean days that followed. In this disease as in the other deficiency diseases we experienced, the value of processed foods for relieving deficiency states was far greater than we had anticipated.

Nicotinic acid was available in limited amount for the first time. In the stubborn cases in whom symptoms persisted even after the improvement in diet, 25 to 100 mg was usually sufficient to obtain relief of symptoms. However, a few were only partially or temporarily controlled on as much as 500 mg per day. The latter were usually of a blonde or sandy complexion.

Nicotinic acid proved to be an effective aid in the treatment of oral sepsis particularly trench mouth. The cheilosis, the para-alar seborrheic dermatitis and the dermatitis of the malar eminences usually attributed to riboflavin deficiency were found to respond to nicotinic acid as well as to yeast therapy. This again brings out the difficulty of classifying lesions according to specific vitamin lack and the suggestion of a high degree of overlapping effect of the vitamins of the B complex.

Those patients who were shown to have decreased or absent free hydrochloric acid by gastric analysis were treated with dilute hydrochloric acid orally and nicotinic acid, thiamine and liver extract parenterally. Laboratory studies in-

dicated improved gastric secretion in some cases but treatment was generally of only symptomatic value suggesting irreversible damage.

In the spring of 1943 we prepared an infusion of rice bran or millings hoping to extract elements of the B complex. This infusion was then used in place of the water in our yeast culture. Unfortunately, soon after, the issue of sugar was stopped, the general ration cut to its previous low level and we were no longer allowed to have the rice bran. It was impossible to continue either the yeast culture or the preparation of the infusion.

With the decrease in the ration and the exhaustion of the Red Cross foods cases of pellagra began to recur and we found the pure vitamin preparation to have much less effect in cases of comparable severity. The stomatitis and dermatitis would improve slightly as long as a man was maintained on a high nicotinic acid intake, only to relapse promptly when the vitamin was withdrawn. Our supply of nicotinic acid dwindled rapidly and we were again forced to treat the more severe cases at the expense of those less severe.

The incidence and severity of the disease had again reached serious proportions by the fall of 1943. Fortunately, in December, 1943, another Red Cross shipment of food and medicine arrived. A quantity of nicotinic acid tablets and multivitamin capsules containing 10 mg of nicotinic acid was included. Every man received 1 multivitamin capsule daily and all those with signs of pellagra were given nicotinic acid in amounts similar to those used the year before.

Again we noted the increased effect of pure vitamin preparations when the diet was more nearly adequate in protein and B complex content. By conserving and using the vitamin preparations sparingly we were able to continue distribution of 1 multivitamin daily until the time of our release by the American Forces in January, 1945.

Early in 1944 the ration was cut to less than 1200 cal. and during the year the ration was gradually cut more. By June

those doing hard physieal work were being issued less than 1000 cal. and the others less than 800 cal. per day. The diet was augmented some by individual gardens.

Throughout the year chronic mild pellagra continued to affect 25% to 50% of the prisoners despite daily administration of 1 multivitamin capsule. Relapses often followed diseases like dengue, malaria, or dysentery in which fever, anorexia and vomiting were prominent symptoms. Remissions followed increase in multivitamin intake and nicotinic acid in doses of 25 to 100 mg per day. Small quantities of fruit, cocoanut, mongo beans, or carabao meat secured from the Filipinos were given as an extra ration to the worst patients.

We observed that once a man had developed pellagra he was much more susceptible to the disease and more resistant to treatment under the same dietary conditions than the man who somehow had escaped the severe form of the disease in 1942.

The morbidity from pellagra in 1944 was less serious than we had anticipated in view of the very inadequate diet. We concluded that the daily multivitamin, although not preventing the appearance of pellagra, tended to prevent the severe fulminating form seen in 1942.

SUMMARY

1. Pellagra was observed in American troops living for 3 years on a deficient diet in Japanese Prison Camps in the Philippine Islands.

2. Men developed pellagra while on a diet low in calories, high in carbohydrates and lacking in animal proteins, fresh fruits, and vegetables.

3. Pellagra appeared in epidemic proportions after 6 months on a deficient diet.

4. The signs and symptoms that were observed are described. The clear separation of certain dermal and neurological lesions according to specific vitamin lack was difficult.

5. Processed foods, a crude yeast culture, and pure vitamin preparations were effective in controlling pellagra. Pure vitamin preparations were more effective when the diet was more adequate.

6. The daily multivitamins utilized during 1944 reduced the incidence of the severe pellagra seen in 1942 and 1943.

PHOTOSENSITIZED OXIDATION OF ASCORBIC ACID IN URINE AND BLOOD SERUM

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Martini reported in 1934 the photodynamic action of flavins on ascorbic acid solutions. Kon and Watson ('36) and Kou ('37) published data showing that the ascorbic acid in both raw and pasteurized milk was reversibly oxidized on exposure to light, that short wave lengths of visible light were responsible, that the biologically active product formed underwent further decomposition without the agency of light and that oxygen was required for the reaction. Hand, Guthrie and Sharp ('38) stated that since dissolved oxygen and ascorbic acid solutions are colorless and do not absorb light, another substance must be involved in this photosensitized reaction. They showed the substance to be riboflavin and claimed it to be the sole agent responsible for the oxidation of ascorbic acid in milk in a pH range from 5 to 9.6. Hand and Greisen ('42) demonstrated that riboflavin and light oxidized ascorbic acid in solutions at a range in pH of 3.9 to 7.7 and compared this oxidation with that caused by copper and by cucumber oxidase.

More recently data have accumulated showing the loss of riboflavin in milk on exposure to visible light (Peterson, Haig and Sbaw, '44; Ziegler, '44; Stamberg and Theophilus, '44). Holmes and Jones ('45) have called attention to the simultaneous loss of reduced ascorbic acid. The effect of various lighting conditions on riboflavin solutions has been investigated by DeMerre and Brown ('44).

During a study of human requirements of ascorbic acid in this laboratory it was observed that reduced ascorbic acid in urine, preserved at pH 2 to 3, also was destroyed on exposure to light. Even on low riboflavin intakes some excretion of riboflavin occurs (Sebrell, Butler, Wooley and Isbell, '41; Hagedorn, Kyhos, Germek and Sevringhans, '45). Riboflavin and possibly other fluorescent constituents of urine can cause this photosensitized oxidation of the reduced ascorbic acid. Since blood serum contains the components necessary for this photochemical activity, the effect of light on it has been tested also.

Data are presented to show that a photosensitized oxidation of reduced ascorbic acid occurs in urine preserved at pH 2 to 3, that after the oxidation dehydroascorbic acid is present and that the reaction depends upon the presence of dissolved oxygen. Riboflavin is an active agent in this change since increasing amounts accelerate the reaction. It is shown, too, that the concentration of reduced ascorbic acid in blood serum is decreased on exposure of the serum to light.

EXPERIMENTAL

Urine

Reduced ascorbic acid in urine was determined by the method of Evelyn, Malloy and Rosen ('38), and the total ascorbic acid by that of Roe and Kuether ('43). At the end of each exposure period the samples were stored in the dark and all analyses were made at the end of the total exposure time. The urine samples were collected directly after taking ascorbic acid in order to produce a urine of sufficiently high ascorbic acid content to show the progressive destruction during a 3-hour period. The samples were brought to pH 2.5 by the addition of glacial acetic acid. An ascorbic acid intake of 100 mg per day by a normal individual results in a 24-hour urine excretion of approximately 3 mg per 100 ml.

The effects of wave length and intensity of light were not studied. However, in order to have a linear relationship be-

tween loss of reduced ascorbic acid and time a constant light source was necessary. A 300-watt tungsten filament bulb furnished an intense light but evidently not of optimum wave length, since less intense skyshine caused a more rapid destruction (table 1). The removal of oxygen by dry ice checked the destruction (table 2). The effect of temperature was not investigated.

TABLE 1
Photochemical oxidation of ascorbic acid in urine.¹

TIME OF EXPOSURE	CONSTANT LIGHT ¹ 500 FOOT-CANDLES 23-25°C		SKYSHINE, ² 150 INCREASING TO 300 FOOT-CANDLES 24-29°C	
	Reduced ascorbic acid	Total ascorbic acid	Reduced ascorbic acid	Total ascorbic acid
Min.	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
0	7.85	7.85	7.85	7.85
30	7.45	7.55	7.05	8.00
60	6.95	7.70	6.50	8.00
90	6.55	7.65	5.55	8.25
120	6.05	7.65	4.80	8.15
150	5.60	7.65	3.90	8.15
180	5.25	7.55	2.55	7.85

¹Urine, pH 2.5, exposed in 25 ml quantities.

²Constant light furnished by 300-watt tungsten filament bulb at 15 inches, 500 \pm 50 foot-candles, depending on angle of incidence.

³Skyshine, exposure in an open north window, tree-shaded. Light measured by Weston meter.

TABLE 2
Effect of oxygen removal (dry ice added for 45 minutes prior to exposure) on the photochemical oxidation of ascorbic acid in urine.¹

TIME OF EXPOSURE	CONSTANT LIGHT ¹ 500 FOOT-CANDLES 35-36°C		SKYSHINE, ² FLUCTUATING BETWEEN 100 AND 140 FOOT-CANDLES 28-29°C	
	Reduced ascorbic acid	Total ascorbic acid	Reduced ascorbic acid	Total ascorbic acid
Min.	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
0	9.60	9.90	9.60	9.90
60	9.50	9.30	9.35	9.65
120	9.40	9.80	9.05	10.0
180	9.45	9.20	8.75	9.65

¹See footnotes, table 1.

Although increasing amounts of riboflavin have been shown to accelerate the destruction of reduced ascorbic acid (table 3), riboflavin is not the only factor in urine which may so act. It is a normal constituent and is typical of other fluorescent and dye substances which may accept hydrogen from reduced ascorbic acid.

TABLE 3

Effect of added riboflavin on the photochemical oxidation of ascorbic acid in urine.¹

RIBOFLAVIN ADDED	CONSTANT LIGHT, ¹ 60 MIN. EXPOSURE	
	Reduced ascorbic acid	Total ascorbic acid
mg	mg/100 ml	mg/100 ml
0	5.70	6.70
0.08	5.20	6.75
0.16	4.95	6.95
0.32	4.60	6.90
0.64	4.10	6.95

¹ See footnotes, table 1.

Blood

Table 4 presents data illustrating the effect of light on samples of the same human blood serum, clear and hemolyzed, exposed in centrifuge tubes in the 0.1 ml quantities used for analysis. At the end of the exposure periods the tubes were stored in the dark. Protein precipitation and analysis for reduced ascorbic acid by the Mindlin and Butler ('38) micro-method followed at the end of the total exposure time. In addition to the exposure to the constant light used, samples were also exposed to a white fluorescent 15-watt light, at 12 inches.

While these data are not analogous to those given for urine since there are no total ascorbic acid values, they show the loss of reduced ascorbic acid in blood serum on exposure to light and emphasize the necessity in this analysis for immediate precipitation of protein and removal with it of the effective catalyzing agents.

The lowered reduced ascorbic acid content of hemolyzed blood serum is well recognized. These data suggest that this loss has occurred sometime prior to exposure and the rate of loss in these slightly hemolyzed samples thereafter is approximately the same as for the clear serum. The degree of hemolysis may alter this rate of change. There have been indications throughout the work that the red color liberated in severe hemolysis protects the reduced ascorbic acid present from the photosensitized oxidation.

The pH of blood serum makes the alteration of reduced ascorbic acid in vitro in this medium more complicated than

TABLE 4

Reduced ascorbic acid of human blood serum exposed to constant light and white light.

TIME Min.	CLEAR SERUM		HEMOLYZED SERUM	
	Reduced ascorbic acid		Reduced ascorbic acid	
	Constant light ¹	White light ²	Constant light	White light
	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
0	1.12	1.12	0.86	0.82
60	0.73	.	0.58	..
120	0.41	0.40	0.25	0.24
180	0.14	.	0.0	..
240	0.13	0.20	0.0	0.0

¹ See footnote 2, table 1.

² 15-watt Mazda fluorescent white light at 12 inches.

that in the acidified urine. There is in all probability a secondary loss, due to the pH, superimposed on the light-catalyzed reaction. The data do demonstrate, however, an oxidation which is stopped when light is removed.

In the course of the blood work it was found that the light from a 15-watt white fluorescent light tube at 12 inches was as effective in oxidizing reduced ascorbic acid as was that from the 300-watt tungsten filament bulb used as a constant light source. The white light and daylight lamps radiate a greater proportion of the light effective in this light-

sensitized reaction (ultraviolet and short visible rays) than does the more intense tungsten filament bulb used as a source of constant light (Barnes, Forsythe and Karash, '39).

The above findings indicate that during the routine preparation of a sample for determination of reduced ascorbic acid by indophenol methods, a significant loss may occur both in urine and blood serum. It is important, therefore, to guard against exposure to light samples which are being analyzed for reduced ascorbic acid. The similarity of conditions producing the photochemical destruction of riboflavin and reduced ascorbic acid suggests an interdependence of these two compounds leading to their mutual alteration and the need for like precautions in the analysis of each when the other is present.

SUMMARY

1. The photosensitized oxidation of reduced ascorbic acid in urine preserved at pH 2 to 3 has been shown to take place.
2. The ascorbic acid is present in the urine after oxidation as dehydroascorbic acid.
3. The reaction depends upon the presence of dissolved oxygen.
4. The reaction is accelerated by increasing amounts of riboflavin.
5. A loss of reduced ascorbic acid occurs in blood serum exposed to light.

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THE INFLUENCE OF RIBOFLAVIN CONSUMPTION ON ITS CONCENTRATION IN HENS' EGGS

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TWO FIGURES

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This paper is a report of a series of experiments designed to investigate the efficiency with which hens transfer riboflavin from their feed to the eggs and the influence of the level of the dietary riboflavin on this efficiency. Data indicating the effects of breed and variety, variations between individuals and with the same individual and the variation over a period of several months of experimental feeding are included. The investigation was undertaken to determine the practicability of introducing additional riboflavin into the human dietary through the medium of eggs laid by hens fed the vitamin.

The level of riboflavin in the feed has been shown to play an important part in the hatchability of the eggs produced. Lepkovsky, Taylor, Jukes and Almquist ('38), Hunt, Winter and Bethke ('39) and Engel, Phillips and Halpin ('40) have reported a direct variation of the riboflavin content of the eggs with that of the ration of the laying birds. Norris and Bauernfeind ('40) found that the riboflavin concentration of the eggs could be increased to a maximum level beyond which increases in dietary riboflavin had no effect. This level was 800 to 1000 μg of riboflavin per 100 gm of ration. No reports

¹ Under the direction of Alan Brown, M.D., F.R.C.P. (Lond.).

have appeared to date of experiments on the balance between riboflavin intake and output.

Sherman and Lanford ('38) reported that, on the average, a whole egg contained 65-70 Sherman-Bourquin units. They also state that one Sherman-Bourquin unit was equivalent to 3-5 μg . From the result of Hunt, Winter and Bethke ('39) it would appear that the riboflavin content of eggs laid by hens fed the usual type of ration was about 200 to 250 μg . The maximum values of Norris and Bauerfeind ('40) calculated for the whole egg were approximately 170 μg . Peterson, Dearstyne, Comstock and Weldon ('45), using a fluorometric determination, found great differences between birds in the riboflavin content of their eggs. The average value for whole eggs was 3.2 μg per gm (about 163 μg per egg). The most recent compilation of the U. S. Department of Agriculture and the National Research Council ('45) gives a riboflavin value of 3.4 μg per gm of fresh whole egg (about 173 μg per egg).

METHODS

The ration samples were analyzed by the microbiological procedure of Snell and Strong ('39) with a modified extraction method that had been previously used for the assay of cereals (Jackson, Doherty and Malone, '43). Occasional samples of the rations containing the higher levels of riboflavin were also assayed by a suitable fluorometric procedure with satisfactory checks.

The eggs were assayed by a modified Hodson and Norris ('39) type of fluorometric procedure.² All the eggs comprising a sample were broken out and mixed in a Waring blender. A weighed sample of the melange was mixed with two parts of water to give an even suspension that could be pipetted readily. Ten ml of this suspension were mixed with 10 ml of water and 20 ml of 0.2 N HCl in a 125 ml flask. After steaming for 15 minutes and autoclaving for another 15 min-

²This method was developed by D. M. Young, Department of Animal Nutrition, Ontario Agricultural College, with the cooperation of the Ontario Research Foundation, Toronto. The technique was modified in some minor respects.

utes at 15 pounds pressure, the flask was cooled and the contents adjusted to pH 5.0 with 0.5 N NaOH, using a glass electrode pH meter. The mixture was diluted to 50 ml with water and filtered. The filtrate was carefully adjusted to pH 6.5, using 40% NaOH. A 15 ml aliquot of this water extract was diluted to 50 ml with acetone and kept over night at 4°C. It was filtered while cold through a Whatman no. 3 filter paper. A perfectly clear filtrate was obtained. Two 15 ml aliquots of this filtrate were pipetted into each of two test tubes; 0.10 ml of a standard riboflavin solution containing 20 µg per ml was added to one tube and mixed. The total fluorescence of each solution was then determined in a Pfaltz and Bauer fluorometer. The difference between the readings, corrected for the small dilution caused by addition of the standard, gave the fluorescence equivalent to 2 µg of riboflavin. The blank was obtained by adding 0.2 ml of fresh, ice-cold 5% $\text{Na}_2\text{S}_2\text{O}_4$ solution in 5% NaHCO_3 to the cuvette. The reading was taken immediately after mixing, before any oxidation of the riboflavin by the air could take place. Recoveries of added riboflavin were from 95 to 99%. Checks were consistently within $\pm 3\%$, and the results obtained by this method agreed closely with those obtained by a more elaborate procedure, a modification of the Najjar ('41) procedure. All riboflavin assays were conducted in a dark room under red illumination.

Experiment no. 1

This experiment was designed to study the riboflavin content of eggs laid by birds maintained in the conventional type of house and fed practical rations supplying different levels of riboflavin. In addition, since Bird and Marvel ('43) had reported that ingestion of feces eliminated the effect of low riboflavin rations on hatchability, it was considered advisable to include birds kept in batteries, to eliminate coprophagy insofar as possible. Accordingly, Barred Plymouth Rock pullets were grouped in six pens and in two

groups of individual hen batteries. The number of birds in each pen varied during the course of the experiment, due to mortality, but did not drop below twenty-five at any time.

Four rations, numbered 1 to 4, were designed to contain, respectively, 800 μ g, 1200 μ g, 1600 μ g and 2000 μ g of riboflavin per pound. A basal mash of the following composition, ground yellow corn 10.0, rolled oats 12.0, ground oats 10.0, wheat bran 9.0, wheat shorts 4.0, dehydrated alfalfa meal 4.0, dehydrated cereal grass 2.0, meat meal 5.0, fish meal 2.5, soy bean oil meal 8.0, iodized salt 1.0, oyster shell 2.5, bone

TABLE 1
Composition of rations (lbs.).

INGREDIENT	RATION			
	1	2	3	4
Basal mixture	71.00	71.00	71.00	71.00
Casein (vitamin-free)	1.75
Buttermilk powder	5.00	5.00	5.00
Soybean oil meal	1.25	1.25	1.25
Barley	15.25	10.75	10.75	10.75
Wheat	12.00	11.50	8.00	4.25
Riboflavin pre mix ¹	0.0	0.50	4.00	7.75

¹ This was a mixture of 100 pounds of ground wheat and 2.0 gm of synthetic, crystalline riboflavin. The riboflavin was furnished by Merck and Company, Limited, Montreal.

meal 0.5, fish liver oil (3500 A, 400 D) 0.5, and anhydrous manganous sulphate 0.025 pounds, was used. The composition of the rations is shown in table 1. Vitamin-free casein was used to supply milk protein in ration no. 1, in an amount equivalent to that supplied by buttermilk powder in the other rations. The crude protein content of these rations was equalized at approximately 15%. The common practice of feeding scratch grain with laying mashes of this type was not feasible in this experiment as consumption of uniform proportions of mash and grain was desired. Therefore, ground

scratch grain was mixed with the mash in the proportion³ of 1 of grain to 1.3 of mash in all groups.

Pen no. 1 and Battery no. 1 received ration no. 1, pen no. 2 received ration no. 2, pen no. 3 and Battery no. 2 received ration no. 3 and pen no. 4 received ration no. 4. For comparative purposes two additional groups were included. Pen no. 5 received a commercial laying mash and no. 6 received a commercial hatching ration. Scratch grain was incorporated with both rations as above. Oyster shell and grit were supplied to all groups *ad libitum*, and the amounts consumed were recorded.

The riboflavin content of each of the complete rations was determined by assays of samples of each new batch as prepared at about 2-week intervals. Ration no. 1 averaged 964 μg per pound over the course of the experiment, as determined by assay. Ration no. 2 assayed 1350 μg per pound; Ration no. 3 assayed 1669 μg per pound, while Ration no. 4 assayed 2301 μg per pound. The commercial laying mash and the commercial hatching mash, each mixed with ground grains in the proportion 1.3:1, averaged 980 and 1390 μg per pound, respectively. The recommended allowances set forth by the National Research Council ('44) are not less than 800 μg per pound for egg production and 1300 μg per pound for hatching rations. The actual levels of riboflavin fed, as determined by assay, are shown graphically in figure 1.

An initial sample of eggs was collected at the start of the experiment and thereafter at weekly intervals for 4 weeks. At this time the riboflavin content of the eggs appeared to have become reasonably constant for each group. Samples were then collected at intervals of about 4 weeks. Each sample consisted of about forty eggs and was representative of all eggs laid over a 3-day period. The concentration of riboflavin found in these samples, expressed as μg per egg is illustrated in figure 1.

³This proportion was determined by examination of the records of actual relative consumption for some years of mash and grain by birds of similar breeding fed on the same type of rations.

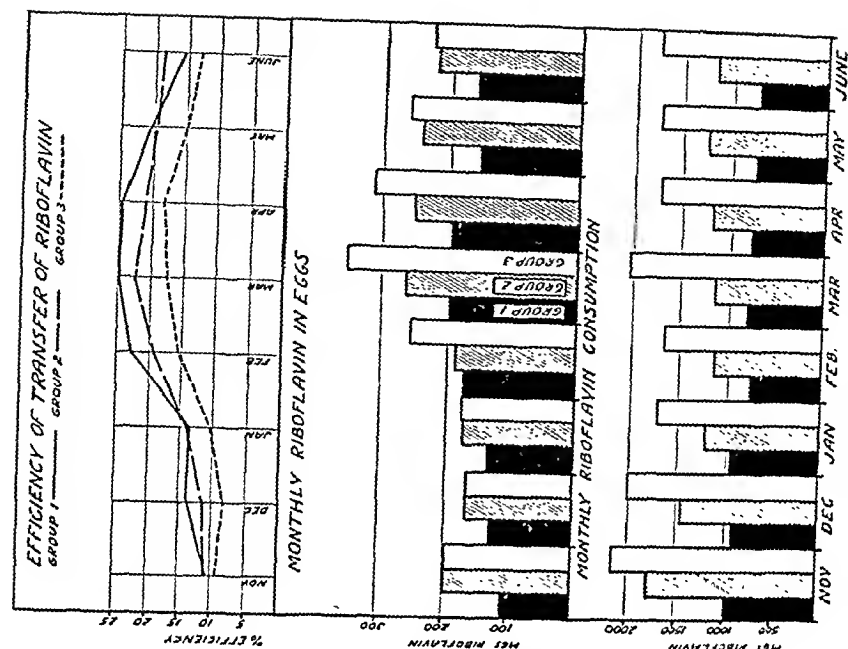


Fig. 2 The variations in riboflavin consumption, riboflavin recovered in the eggs, and efficiency of transfer from the feed to the eggs over an 8-month period.

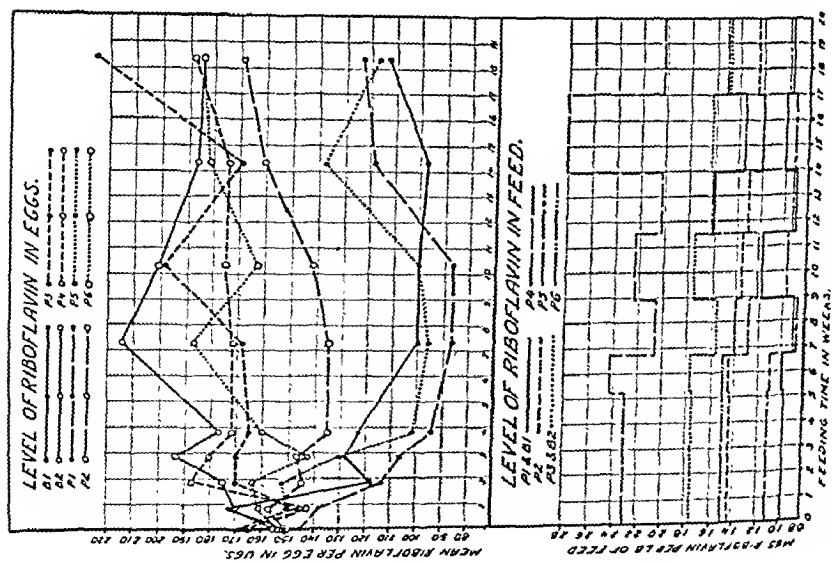


Fig. 1 The relation between the concentration of riboflavin in the chicken feed and the concentration found in the eggs.

An initial rapid adjustment of the riboflavin level in the eggs took place and was reasonably complete in a period of 3 to 4 weeks after beginning the experimental rations. After this initial adjustment Battery 2, and Pens 3, 4 and 6 formed a broad group with fluctuating values ranging from 160 to 225 μg per egg, averaging about 185 μg per egg. Battery 1, Pen 1 and Pen 5 formed a lower group, averaging about 100 μg per egg. Pen 2 was midway between these groups. The proportion of dietary riboflavin recovered in the eggs was calculated for each pen and battery for the period from the fourth to the eighteenth week on the experimental rations. The total riboflavin consumed was calculated from the total feed consumption and the average level of riboflavin in the feed. The total riboflavin recovered was calculated from the total egg production and the average amount of riboflavin per egg for the period. Table 2 shows the results of these calculations.

TABLE 2

The riboflavin balance during the period from the fourth to the eighteenth week of experimental rations.¹

PEN	TOTAL EGG PRODUCTION	AV. WT. EGG CONTENTS	TOTAL FEED CONSUMPTION	AVERAGE RIBOFLAVIN		TOTAL RIBOFLAVIN		RIBOFLAVIN FROM FEEDS RECOVERED IN EGGS
				In feed ²	In eggs	Intake in feed	Output in eggs	
		gm	lb.	$\mu\text{g}/\text{lb}$	$\mu\text{g}/\text{egg}$	mg	mg	%
1	1300	51.5	734	985	105	723	136.5	18.9
2	1509	51.1	704	1310	148	922	223.5	24.2
3	1649	52.9	838	1600	178	1340	294.0	21.9
4	1162	50.8	625	2230	178	1395	207.0	14.8
5	1129	50.6	847	940	112	796	126.5	15.9
6	1808	52.1	983	1380	179	1360	324.0	23.8
Battery 1	1443	51.8	569	985	103	560	149.0	26.6
Battery 2	1429	54.4	593	1600	197	950	281.5	29.6

¹ March 15 to June 15.

² Note that these average values are for the feed consumed during the fourth to eighteenth week only, while figures reported in the text are for the entire experiment.

There was a maximum percentage recovery of the riboflavin in the eggs from birds maintained in pens when the riboflavin content of the feed was about 1300 μg per pound. It is probable that the hen has a definite requirement for body maintenance and normal activity. Hence at lower concentrations of intake, a higher proportion of this intake will be required for maintenance and activity. However, there appears to be a limit to the ability of the bird to efficiently transfer riboflavin to the egg when the available surplus increases. This finding corroborates the report of Norris and Bauernfeind ('40) that there is a maximum level of intake beyond which no increase in the riboflavin content of the egg is achieved. However, the Cornell workers reported this dietary level as 3600 to 4500 μg per pound of ration, whereas this level in our work was about 1400–1600 μg in the case of the birds housed in the conventional manner. An average concentration of 179 μg of riboflavin per egg was the maximum obtainable even when the dietary level was raised to 2230 μg per pound.

The highest recoveries of riboflavin were made by the birds maintained in batteries. This greater recovery in Battery 1, at the level of 985 μg of riboflavin per pound of feed, was entirely due to the production of a greater number of eggs per pound of feed consumed, as the concentration of riboflavin was the same in the eggs from Pen 1 and Battery 1. Raising the level of dietary riboflavin to 1600 μg per pound (Pen 3 and Battery 3) resulted in a greater increase of the level of riboflavin in the eggs from the battery than from the corresponding pen. This greater recovery for Battery 2 resulted from both a greater egg production per pound of feed consumed and a higher concentration per egg. The activity of birds in batteries is less than that of birds housed in pens and this may be responsible, in part at least, for the more efficient egg production per pound of feed consumed.

Since the riboflavin content of the eggs laid by the birds in batteries was as high as, or higher than, those laid by birds fed the same ration but housed in pens, it would appear

that coprophagy does not increase the riboflavin content of the eggs laid.

Experiment no. 2

The opportunity was taken during the foregoing experiment to investigate the variation in performance of individual hens. About twelve hens were selected from each of Pens 1, 2 and 3 and five collections of individual egg samples were made. Each sample consisted of all the eggs laid by one bird for a week. The maximum, minimum and average riboflavin contents of the eggs of these birds are listed in table 3. A considerable range was observed for each bird. This range in riboflavin content of eggs laid by any individual bird is much wider than that observed in other studies carried out over shorter periods at the Ontario Agricultural College.⁴ There is also evidence of variation between individuals in ability to transfer the vitamin from the feed to the egg as reported by Peterson et al. ('45).

Experiment no. 3

After completion of the foregoing experiments and examination of the results, it was decided to repeat the study, expanding its scope by extending it over a longer period of time and elevating the upper level of riboflavin in the feed. Accordingly, 225 Barred Plymouth Rock pullets were divided into three groups, each group consisting of three pens of twenty-five birds each. The pens were kept up to strength throughout the study by replacement of any losses by birds of similar breeding. Eggs laid by these replacements were not used for assay purposes until the bird had been on the ration for at least 4 weeks. The birds were fed an "all mash" ration made up with the same formula as that previously used. The mean value of the assays of all samples of feed for Group 1 for the period of study was 1185 μg per pound of feed, the mean for Group 2 was 1870 μg per pound and that for

⁴ Unpublished data, Department of Animal Nutrition and Department of Poultry Husbandry.

TABLE 3
Riboflavin content of egg samples from individual hens. Experiment no. 2.

PEN NO. 1 RIBOFLAVIN — 985 µg/LB. ¹			PEN NO. 2 RIBOFLAVIN — 1310 µg/LB. ¹			PEN NO. 3 RIBOFLAVIN — 1600 µg/LB. ¹		
Bird no.	Riboflavin/egg		Bird no.	Riboflavin/egg		Bird no.	Riboflavin/egg	
	Range µg	Average µg		Range µg	Average µg		Range µg	Average µg
903	32-108	68	943	84-163	135	985		123
904	76-143	110	944	150-182	166	986	143-176	160
906	85-108	99	946	94-152	118	987	183-234	214
910	88-129	114	947	127-162	149	989	131-155	142
911	90-164	124	948	99-168	135	994	179-235	192
912	64-151	106	949	153-220	193	993	133-191	157
915	74-119	92	951	97-171	127	996	170-225	192
916	74-133	101	952	101-195	149	997	139-171	149
917		92	954	133-200	161	998	100-163	131
919	60-91	75	958	101-185	149	999	119-133	128
920	82-167	107	960	110-245	178	1401	121-171	149
921	91-119	109	961	107-145	124	1404	138-194	173
923	62-101	83	962	81-135	115	1402	150-171	161
924	115-135	125	967		114	1405	127-178	147
929	83-106	95	968		80	1410		212

¹ Average riboflavin content of the feed after the initial adjustment period.

Group 3 was 2560 μg per ponnd. The study was maintained for a period of 8 months, from November to June inclusive. A large representative sample of eggs from each group was collected over several days and assayed by the fluorometric procedure. The results are illustrated graphically in figure 2.

The monthly riboflavin consumption for each group was calculated from the feed consumed each month and the riboflavin assays of the batches of feed used during the month. The monthly output in the eggs for each group was calculated from the total egg production for each month and the riboflavin assay of the egg sample for that month. The efficiency of transfer of riboflavin from the feed to the eggs for each month was calculated from the riboflavin recovered in the eggs each month and the riboflavin consumption.

The main point of interest revealed by this study was the relatively large increase in total riboflavin output in the eggs during February, March, April and May. This increase was due largely to an increase in total egg production during these months, but the concentration of riboflavin in the eggs followed the same general variation in a lesser degree. This cycle was reflected in the efficiency curves.

The larger amount of dietary riboflavin supplied to Group 2 as compared to Group 1 resulted in an approximately proportional increase in the riboflavin output in the eggs for each month, with the exception of February. On the other hand, the increased dietary riboflavin consumed by Group 3 compared to Group 2 did not result in any significant increase in riboflavin output in the eggs during November, December, January and June but it did produce a definite increase in riboflavin output in the eggs in February, March, April and to a lesser extent, in May. This increase was the result of a higher concentration of riboflavin in the eggs and not the result of a gain in egg production by Group 3 over Group 2. Hence it appears that during the period of maximum egg production there was also an elevation of the upper level of dietary riboflavin that will influence the concentration of riboflavin in the eggs. The upper effective level of dietary ribo-

flavin was ordinarily about 1600 μg per pound of feed but it was more than 1870 μg per pound of feed during the period of maximum egg production and may have reached as high a level as 2500 μg per pound or even higher. It is possible that higher levels of riboflavin, had they been used, would have been found to raise the concentration of the vitamin in the egg still more. This finding, which was not obvious in the first experiment, probably explains, in part at least, the discrepancy between our original observation and that of the Cornell workers with regard to the maximum effective level.

Experiment no. 4

As a preliminary investigation, egg samples were also collected from a number of breeds and varieties of poultry maintained in pens and fed on the commercial hatching ration used in the first experiment. This ration had an average

TABLE 4

Riboflavin in eggs from different breeds and varieties of poultry fed same ration.

BREED AND VARIETY	AVERAGE WEIGHT EGG CONTENTS	RIBOFLAVIN	RIBOFLAVIN
	<i>gm</i>	$\mu\text{g/gm}$	$\mu\text{g/egg}$
Barred Plymouth Rock	52.5	3.21	168
White Plymouth Rock	48.6	3.03	147
New Hampshires	53.6	3.15	168
White Wyandottes	51.2	3.45	177
White Chanteclers	54.0	3.41	184
New Ontario ¹	47.7	2.91	139
Jersey Black Giants	51.6	3.38	174
Light Sussex	53.0	3.68	195
Light Sussex (yellow skinned) ²	52.6	3.36	177
Black Langshans	57.0	3.71	211
White Leghorns	54.2	3.65	198
Brown Leghorns	47.3	3.43	162

¹ This breed was developed at the Ontario Agricultural College in 1910. The foundation breeds were Light Sussex, White Laced Cornish and Light Red Brahmas.

² This was a strain of Light Sussex selected for yellow skin. The Light Sussex breed normally has white skin.

riboflavin content of 1380 μg per pound but feed consumption records were not available for the particular period of the test. All egg samples were collected at the same time and consisted of at least thirty eggs per sample with the exception of the Black Langshans' sample which consisted of only seven eggs. The assays are shown in table 4.

The riboflavin values of the eggs were reasonably constant, although the suggestion of a breed and/or variety difference does exist. However, it is unlikely that any true breed or variety difference that might be shown by a more complete investigation of these breeds and varieties would prove to be of practical importance.

DISCUSSION

The primary objective of this investigation, as previously stated, was to discover the feasibility of supplying riboflavin in the human diet by feeding the vitamin to hens and recovering it in the eggs. Some calculations based on the data given in table 2 show that such an addition would be comparatively efficient if the proper concentrations of riboflavin in the feed were observed. The usual type of laying ration contains about 1000 μg of riboflavin per pound and was represented by Pens 1 and 5 in the first experiment. The eggs from these pens averaged about 110 μg of riboflavin per egg. This was raised to 148 μg per egg in Pen 2 and to 179 μg per egg in Pen 6 by raising the riboflavin in the feed to 1310 and 1380 μg per pound, respectively, that is, to the usual hatching ration level. A consideration of Pens 1 and 2 in the first experiment shows that each pound of feed in Pen 1 contained 985 μg of riboflavin and 18.9% or 186 μg of this was recovered in the eggs. Each pound of feed in Pen 2 contained 1310 μg of riboflavin and 24.2% or 317 μg of this was recovered in the egg. Hence, from an addition of 325 μg of riboflavin per pound of feed, 131 μg were recovered in the eggs. Thus 40.3% of the extra riboflavin required to raise the dietary level from 985 to

THE URINARY EXCRETION OF RIBOFLAVIN BY COLLEGE WOMEN¹

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TWO FIGURES

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INTRODUCTION

Parsons ('44) and Copping ('45) have reviewed the literature concerned with the metabolism of riboflavin. Except in conditions of extreme deficiency, in which case specific symptoms may be present (Goldsmith, '43), the means of assessing riboflavin nutrition depend upon the urinary excretion of the vitamin. Apparently urinary losses parallel intakes closely until a point is reached at which higher intakes result in larger quantitative excretion but little increase in percentage excretion. The urinary excretion of riboflavin may also depend upon the nitrogen content of the diet (Sarett and Perlzweig, '43). Since there is no recognized depot for riboflavin storage in the body, it is possible that increased destruction occurs at the higher intakes. An adequate or "optimal" intake of riboflavin might be defined as the upper limit of intake at which there is economical utilization of the substance. This study was planned to investigate the use of riboflavin by young women in the higher ranges of intake at which the body retains an economical proportion of the ingested nutrient.

¹ Authorized for publication as Journal no. 830 (n.s.) of the Michigan Agricultural Experiment Station.

² Present address: University of Chicago, Illinois.

SERIES A

This consisted of a study of the urinary excretion of riboflavin by twenty college women on self-selected diets.

Experimental plan

The urinary excretion of riboflavin of twenty college women was studied for a 4-day period. These women were living in their usual college residences and the diet was self-selected. Twenty-four-hour urine samples were collected for the first 3 days. On the fourth morning a 1-hour fasting sample was obtained. At this time the subject was given an oral test dose containing 3 mg of riboflavin and 3 mg of thiamine hydrochloride³ and a light breakfast. Urine was collected for the following 4-hour period and a separate collection of urine was made for the next 19 hours.

The riboflavin content of the urine was determined by the adsorption procedure of Conner and Straub ('41) as modified by Keys ('44). The degree of fluorescence was measured in a photo-electric fluorescence meter.⁴ The instrument was standardized against a solution containing 0.1 μ g of riboflavin per ml.

Results and discussion

The urinary excretions of riboflavin for these subjects are given in table 1. The range of urinary riboflavin for the 3-day period on the self-selected diet was from 0.06 to 0.92 mg in 24 hours. Two students excreted less than 0.10 mg per 24 hours. No riboflavin was demonstrable in the 1-hour fasting sample excreted by the subject for whom the lowest value, 0.06 mg, was obtained in the 3 days on the non-restricted diet. The urinary values following the test dose were correspondingly low for this subject. Fifteen per cent of the supplement was excreted in 4 hours and 20% in 24 hours. The percentage excretion of the test dose excreted in 24 hours was calculated

³ Acknowledgment is made to Hoffmann-LaRoche, Inc., for the supply of riboflavin and thiamine hydrochloride.

⁴ Lumetron, Will Corporation.

from the difference between the urinary value obtained after the administration of the dose and the average excretions for 3 days on the self-selected diet.

The group as a whole excreted from 9 to 57% of the 3-mg test dose in the 4 hours following its administration. In 24 hours after the test dose, 4 of the twenty subjects excreted less than 20% and ten of the subjects excreted more than 30% of the added supplement. The highest excretion was 68%.

TABLE 1

Urinary riboflavin excretion of twenty college women.

CONDITIONS	URINARY EXCRETION OF RIBOFLAVIN	
	2	18
No. of cases		
Average, 3 days on non-restricted diet		
mean, mg per day	0.08	0.49
range, mg per day	0.06-0.10	0.16-0.92
S.D., ¹ mg		0.04
1-hour fasting		
mean, mg per hr.	0.004	0.02
range, mg per hr.	0-0.008	0.001-0.04
S.D., mg		0.005
4 hours after oral test dose		
Mean, mg per 4 hrs.	0.50	0.82
range, mg per 4 hrs.	0.46-0.54	0.26-1.72
S.D., mg		0.10
24 hours after oral test dose		
mean, mg per 24 hrs.	0.76	1.47
range, mg per 24 hrs.	0.67-0.84	0.52-2.80
S.D., mg		0.13

Correlation coefficients

Average excretion for 3 days on non-restricted diet with:

1-hr. fasting excretion — 0.602²

4-hr. excretion after oral test dose — 0.570²

24-hr. excretion after oral test dose — 0.736²

1-hr. fasting excretion with:

4-hr. excretion after oral test dose — 0.338

24-hr. excretion after oral test dose — 0.592²

¹ Standard deviation of the mean.

² Less than one chance in 100 of a fortuitous result.

The average urinary excretion for the 3 days on the self-selected diet was significantly related to (a) the 1-hour fasting excretion, (b) the 4-hour excretion after the oral test dose, and (c) the 24-hour excretion after the test dose. There was also a significant relationship between the 1-hour fasting excretion and the 24-hour excretion after the test dose. The correlation coefficient obtained between the 1-hour fasting test and the 4-hour excretion after the test dose did not possess significance. It is possible that irregular absorption of riboflavin occurs following the oral dosage. Our observations, with this exception, support the conclusion of Oldham and others ('44), that the 1-hour fasting excretion, the 4-hour, and the 24-hour return of a test dose reflect equally well the nutritional status with respect to riboflavin. However the difficulty in determining the small amounts of riboflavin in the 1-hour fasting excretion supports the criticism by Hagedorn et al. ('45) of this technique. For the purpose of the controlled dietary study in Series B, the 24-hour urinary excretion after a test dose was selected for evaluation of the riboflavin status of the individual.

SERIES B

This series consisted of a study of the urinary excretion of riboflavin by fourteen college women on controlled intakes.

Experimental plan

Fourteen college women acted as subjects for this phase of the study. The subjects were in good physical health throughout the experiment as established by physical examinations. The age, height, and weight of the subjects are given in table 2.

The urinary excretion of riboflavin by the subject on her customary, self-selected diet was studied for 6 days. For 6 succeeding days the customary diet of the subject was supplemented daily with 3 mg of riboflavin and 3 mg of thiamine hydrochloride and the urinary excretion of riboflavin was determined. For the following 12-day period the subject re-

ceived the experimental diet only and for two additional periods of 12 days each she received the experimental diet supplemented with riboflavin. On the day following each of these periods the subject was given the experimental diet and a test dose of 3 mg of riboflavin. For 3 days preceding the second and third periods on the experimental diet the subjects returned to a self-selected diet supplemented with 3 mg of riboflavin and 3 mg of thiamine so that a high tissue storage of vitamin was present at the beginning of each experimental period.

TABLE 2

Physical characteristics of subjects.

SUBJECT	HEIGHT	WEIGHT	AGE	SUBJECT	HEIGHT	WEIGHT	AGE
		kg				kg	
BN	5'9"	66.3	23	BY	5'5"	53.0	22
JF	5'6"	68.2	24	GB	5'4"	65.7	20
MJ	5'4"	65.3	22	VB	5'5"	63.1	27
PK	5'7"	60.4	22	LK	5'4"	54.1	23
EM	5'	52.6	24	AC	5'4"	54.3	27
BS	5'	50.5	21	BM	5'2"	45.5	32
EJ	5'4"	54.6	22	RI	5'	54.6	28

Experimental diet. The low riboflavin diet was planned so that menus were repeated every 3 days (table 3). The diet provided from 2100 to 2300 cal. daily. The calorie requirement of each subject was estimated on the basis of her activity, size and food habits. Adjustments in the calorie value of the diet were made by variations in the amounts of riboflavin low foods such as butter, sugar and bread prepared from non-enriched flour. Each serving of food was weighed and it was assumed that the calorie value of the diet was constant for each subject throughout the period of study. The weights of the subjects remained uniform throughout the experiment. The riboflavin content of the milk in the diet was partially destroyed by exposure of the milk to ultraviolet light for 30 minutes with continuous stirring. This treatment produced an undesirable change in flavor but all of the subjects were able to accustom

themselves to the flavor. Daily supplements to the diet were 0.5 gm of tri-calcium phosphate and one-half teaspoon of cod-liver oil. Analyses of the dietary intakes and urinary excretions of thiamine by the subjects will be reported elsewhere; the dietary intake of thiamine was adequate to maintain a constant urinary excretion of thiamine.

TABLE 3
Experimental diet. Total foods included in 3 days.¹

FOOD	WEIGHT	FOOD	WEIGHT	FOOD	WEIGHT
	gm		gm		gm
Farina, cooked	405	Corn, whole kernel	70	Apple sauce	126
Bread, white non-enriched	300	Green string beans	67	Pears	100
Saltines	12	Peas	70	Apple, cooked	132
Spaghetti, cooked	120	Cabbage, shredded	43	Grapejuice	100
Rice, cooked	100	Carrot strips	20	Dates, stoned	25
Milk	732	Celery	20	Sugar cookies	84
Butter	156	Split pea soup	196	Brownies	33
Cream	60	Tomato juice	100	Apple sauce cake	50
Beef, ground, cooked	264	Grapefruit juice	360	Sugar	52
Bacon, cooked	16	Fruit cocktail	200	Jelly	144
Potato	360	Peaches	170	Mayonnaise	14
				Gelatin	24

¹ By analysis: thiamine, 0.62 mg/24 hrs.; niacin, 9 mg/24 hrs.; and riboflavin, 0.79 mg/24 hrs.

Methods. The riboflavin content of the diet was analyzed daily by a modification of the method of Conner and Straub ('41). An aliquot which represented one-fifth of the amount served was taken of each food included in the day's diet exclusive of butter and sugar. The food was stored under 2% acetic acid and this concentration of acid was used for rinsing dishes. Combined aliquots were blended and samples were taken for analyses.

Twenty-four-hour collections of urine were made in amber glass bottles which contained glacial acetic acid. Riboflavin analyses were made on combined aliquots of three successive 24-hour collections which were held in a household electric

refrigerator until the 3-day collections were complete. In the early period of the work, the riboflavin in the urine was determined by a direct fluorometric procedure (Sure and Ford, '42) but this method was found unsatisfactory for urines of low concentration. For samples of urine containing 1.1 mg of riboflavin or more, values obtained by the direct procedure and the adsorption procedure compared closely. The adsorption procedure described under series A was used for all urinary data reported except as indicated in the tables.

Results and discussion

In the preliminary periods, when the food intake was not restricted, the average urinary excretion of riboflavin by the subjects was from 0.14 to 1.07 mg (table 4). This range was similar to that for the subjects in series A and to the range of urinary values reported by Sebrell et al. ('41).

When the self-chosen diet was supplemented with 3 mg of riboflavin daily for 6 days, the excretion of urinary riboflavin was from 31 to 82% higher than for the preceding 6 days when no supplement was given. Since the diets in this period were not controlled, the variations in the per cent of the added

TABLE 4

Urinary excretion of riboflavin by fourteen subjects on self-selected diets and after supplementation of diet with 3 mg riboflavin daily.

CONDITIONS	URINARY EXCRETION OF RIBOFLAVIN	
	Mean	Range
	mg/24 hrs	mg/24 hrs
Self-chosen diet		
First 3 days	0.56 \pm 0.069 ¹	0.12-0.99
Second 3 days	0.62 \pm 0.075	0.17-1.15
Self-chosen diet supplemented with 3 mg riboflavin		
First 3 days	1.96 \pm 0.123	1.24-2.96
Second 3 days	2.30 \pm 0.176	1.41-3.79
Per cent of supplement excreted in urine, 6 days	52 \pm 3.7	31-82

¹Standard deviation of the mean.

riboflavin excreted by the subjects may have represented a change in basal excretion resulting from an increased or lowered amount of riboflavin in the diet. The values for urinary riboflavin during the 6-day supplementation period ranged from 1.24 to 3.79 mg per 24 hours. These values and the estimated percentage excretion of the added riboflavin indicated that the subjects were in a state of riboflavin nutrition which approached saturation, as it may be defined from urinary excretion of the vitamin.

TABLE 5

Average urinary excretion of riboflavin by college women on controlled intakes.

NUMBER OF SUB- JECTS	DIETARY INTAKE OF RIBO- FLAVIN	URINARY EXCRETION OF RIBOFLAVIN			URINARY EXCRETION AFTER 3 MG TEST DOSE FOLLOWING DIETARY PERIOD		
		Mean	Range	Per cent of intake	Mean	Range	Per cent of intake ¹
	mg/24 hrs	mg/24 hrs.	mg/24 hrs.	%	mg/24 hrs.	mg/24 hrs.	%
9	0.79	0.07	0.04-0.10	9	0.84	0.65-1.10	22
3	1.04	0.16	0.14-0.20	15	1.18	1.03-1.38	30
9	1.26	0.13	0.07-0.25	10	1.04	0.62-1.45	27
9	1.62	0.32	0.17-0.44	20	1.23	1.02-1.43	31
4	2.23	1.18 ²	1.11 ² -1.29 ²	53	2.08 ²	2.00 ² -2.17 ²	55
4	2.73	1.31 ²	1.27 ² -1.35 ²	48	2.12 ²	1.95 ² -2.26 ²	56

¹ Urinary excretion of riboflavin
 $\frac{\text{Riboflavin content of basal diet} + 3 \text{ mg riboflavin}}{\text{Riboflavin content of basal diet} + 3 \text{ mg riboflavin}} \times 100.$

² Values determined by direct fluorometric procedure.

The average urinary excretion of riboflavin at each of the different riboflavin intakes is given in table 5. The values for riboflavin intake represent the average intake for each 12-day period. The excretion during the first 3 days of each period was influenced by the preceding days when a riboflavin supplement was given. By the end of 6 days the urinary riboflavin became essentially constant. Therefore, riboflavin excretions for each period were averaged from values for the last 3 days of that period. The mean excretion for nine subjects at an intake of 0.79 mg was 0.07 mg riboflavin per 24 hours, or 9% of the intake. Urinary riboflavin was increased to a mean

value of 0.13 mg per 24 hours when the intake was increased to 1.26 mg although the percentage excretion of the vitamin remained approximately 10% of the intake.

Both the absolute values for urinary riboflavin, mean, 0.32 mg per 24 hours, and the percentage excretion, 20%, were higher at an intake of 1.62 mg than at the lower intakes. A sharp rise in excretion occurred when the riboflavin in the diet was increased to 2.23 mg per day. The mean excretion was 53% of the intake. Gardner et al. ('43), reported that for riboflavin intakes of 7.0 mg, an average of 3.4 mg or 49% of the dietary riboflavin was excreted in the urine. A similar percentage excretion of riboflavin was reported by Keys ('44) for young men who received 11.2 mg per day. For our subjects an intake of 2.23 mg per day apparently resulted in as high a proportionate excretion of riboflavin as would occur on greatly increased intakes.

The validity of the 24-hour excretion of riboflavin following an oral test dose of 3 mg as a measure of riboflavin nutrition was discussed in series A. Again for the group of subjects on controlled dietary intake, the 24-hour excretion of riboflavin following the test dose was dependent upon the previous dietary intake of riboflavin of the individual. Of the fourteen subjects studied, four subjects (BS, BY, GB, VB) showed an increase in urinary riboflavin after the test dose following an intake of 1.2 mg riboflavin and for these subjects the excretion of test dose after an intake of 1.6 mg was approximately the same as after an intake of 1.2 mg. The urinary excretion of riboflavin for successive 3-day periods for each dietary intake and the 24-hour excretion of riboflavin after each test dose were averaged for these subjects and the average values are shown in figure 1. For four subjects (JF, MJ, BN, PK) the increase in urinary excretion of riboflavin after the test dose occurred following a dietary intake of 1.6 mg. For these subjects the upper limit of intake which appeared to maintain tissue stores was not determined but an intake of 1.6 mg seemed to approach or exceed this point. For the subjects AC, RI and LK, approximate tissue satura-

tion apparently occurred between intakes of 1.0 and 2.2 mg per day.

A scatter diagram (fig. 2) was prepared from the data from the study reported here and from values for women of the urinary excretion of riboflavin at known intakes which have been reported in the literature. These include the data reported by Strong et al. ('41), Williams et al. ('43), Davis ('45), Sebrell et al. ('41) and Gardner et al. ('43). The distri-

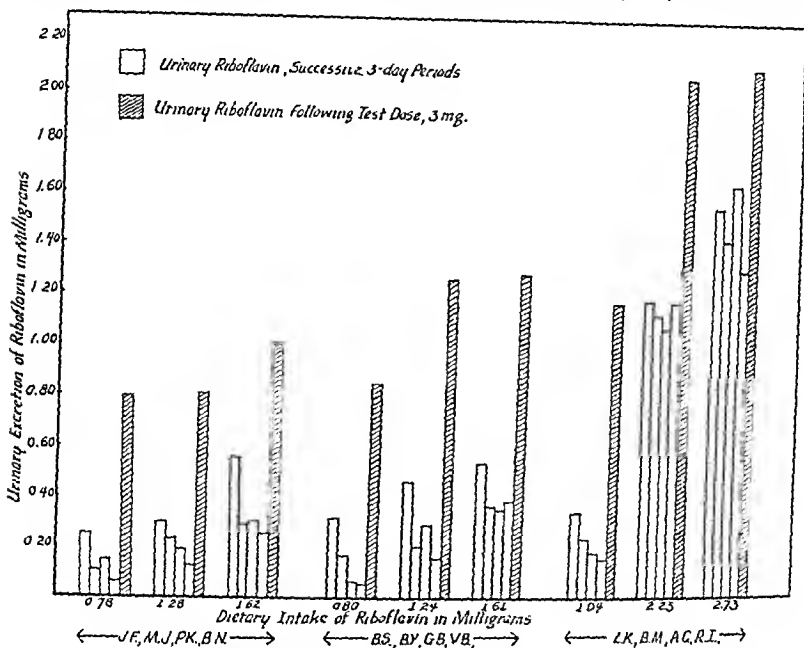


Fig. 1 Excretion of riboflavin of twelve subjects on three dietary intakes and following a 3-mg test dose of riboflavin.

bution of values indicated that a gradual increase in urinary excretion of riboflavin occurred with increasing intakes from 0.50 mg to intakes of from 1.3 to 2.0 mg riboflavin per day. For intakes above this range, a sharp increase in excretion occurred with increased intakes. Using the predicting equation, $y = a + bx$, regression lines were plotted from values from this study, from values from the literature, and from the combined values. For each group of data, one regression

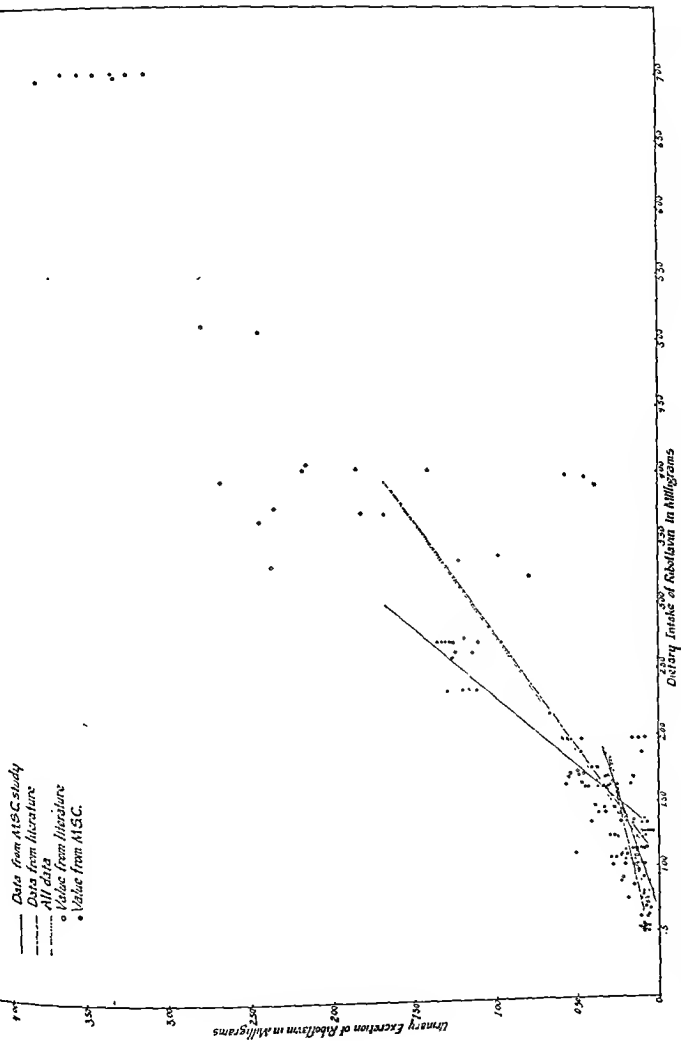


Fig. 2 Urinary excretions of riboflavin at intakes ranging from 0.50 to 7.0 mg; data on women selected from the literature combined with data from this study.

line was plotted for urinary riboflavin at intakes from 0.50 to 2.0 mg and another line from values at intakes of 1.3 mg and above. These limits were selected from the apparent distribution of values on the scatter diagram. The "t" test for significance was applied to the slope of the lines. The slope of the line of values obtained in our study did not differ significantly from the slope of the corresponding line obtained for urinary values reported in the literature. The "t" values for the slopes of the lines predicted from values below 2.0 mg was 1.6; for 1.3 mg and above, the "t" value was 1.08. There was, however, a significant difference between the slope of the lines for values at intakes of 0.50 to 2.0 mg riboflavin per day as compared with the slope of the lines for values at intakes of 1.3 mg riboflavin and above. The "t" value was 3.48.

The points of intersection of lines in the graph lie between intakes of 1.3 and 1.5 mg riboflavin per day. At this area of intake there was an abrupt change in the retention of dietary riboflavin. When the riboflavin content of the diet exceeded an intake of 1.5 mg per day, a rapid rise in excretion of urinary riboflavin occurred with increasing intakes. Under the defined conditions of this experiment and under the varying conditions of study of riboflavin excretion of women as reported in the literature, it would appear that from 1.3 to 1.5 mg riboflavin in the diet represents the upper limit of economical utilization of the vitamin for women with an intake of 2100 to 2300 cal. We have interpreted these findings to indicate that tissue supplies will be adequate under these conditions.

In their study of the riboflavin content of the average American diet, Chelidelin and Williams ('43) reported that diets providing 2500 cal. could be expected to contain 1.4 mg riboflavin and that with enrichment of cereal, the riboflavin could be increased to 1.6 mg. Thus the selection of a sufficient amount of total food for maintenance and the provision of the calcium and protein requirement would supply an adequate amount of riboflavin.

SUMMARY

The urinary excretion of riboflavin by college women was studied on non-restricted diets, after an oral test dose, and on controlled riboflavin intakes.

The urinary excretions for twenty women on self-selected diets ranged from 0.06 to 0.92 mg in 24 hours. The 1-hour fasting excretion, the 4-hour and the 24-hour excretions after an oral test dose were significantly related with the average 24-hour excretion for 3 days preceding the test dose administration.

From three to nine subjects were studied at each of the following riboflavin intakes: 0.79, 1.04, 1.26, 1.62, 2.23 and 2.72 mg daily. The average urinary riboflavin for the last 3 days of each period of controlled diet were as follows: 0.07, 0.16, 0.13, 0.32, 1.18 and 1.31 mg per 24 hours. The percentage of a 3-mg test dose excreted in 24 hours following the periods of controlled intake was, respectively, 22, 30, 27, 31, 55 and 56.

A comparison of the data from this study and the data reported in the literature indicated that 1.3 to 1.5 mg riboflavin per day were adequate for women with a requirement of 2100 to 2300 cal.

ACKNOWLEDGMENT

We wish to express our appreciation for the cooperation of the college women who acted as subjects and to Mrs. Ammanell Jubb for her technical assistance. We are grateful to Dr. W. D. Baten for his advice and help in the statistical analysis of the data.

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BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN

IV. THE ANALYSIS OF FIFTEEN PROTEIN FOODS FOR THE TEN ESSENTIALS¹

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In the third paper of this series (Murlin, Edwards, Fried and Szymanski, '46) reference was made to the analyses upon which were based the mixtures of essential amino acids used in that study in comparison with the proteins which the mixtures simulated. The present report deals with the methods as checked and verified by recovery of the pure synthetic products of Merck and Co. in the case of each acid, and the final results obtained for the proteins selected for the comparison. It must be admitted here again that some of the mixtures compared for biological values with the proteins were not satisfactory because some of the methods used for analysis up to the time the mixtures were compounded had not been adequately checked. Consequently the emphasis in paper III was placed only on the use of mixtures in the later comparisons when the analyses had been more thoroughly tested.

In the description of the methods of assay of the individual acids will be found statements of the average percentage

¹ This work was supported by generous grants from the Nutrition Foundation.

recovery of the pure substances found. Correction has been made in the tables for these recovery figures.

It was confirmed also that lysine, the most vulnerable of these amino acids to destruction by acid hydrolysis (except, of course, tryptophane for which an alkaline hydrolysis always was used) was not destroyed under the conditions which prevailed in the hydrolysis as carried out in this study.

HYDROLYSIS OF FOOD MATERIALS

Treatment of food samples

Meats and fish, freed of extraneous tissue, and egg were treated with dry acetone and ether to remove fat. For example, 150 gm of ground meat were stirred with six volumes of acetone in a Waring blender for 3 minutes. The mixture was allowed to stand for 1 hour with occasional shaking. The solution then was filtered and the residue allowed to dry under suction. As soon as it was dry to the touch the powder was extracted with six volumes of ether in a similar fashion. This process removed the water and most of the fat.

The farinaceous foods, the yeasts and legumes were treated with acetone in the same manner as the animal foods, but for extraction of the acetone dried material the Soxhlet was used and extraction of fat continued over-night. The loss of nitrogen into the acetone from these materials was usually less than 10 mg per 100 gm; but from peanut meal it was 16.7 mg and from Navy bean meal, prepared in the laboratory, it was 112 mg per 100 gm. The loss into anhydrous ether was never more than 3 mg per 100 gm.

One gm of dry protein was placed in a test tube 20 mm by 200 mm. Then 30 ml of 20% HCl was added to the contents in the tube. A 25 ml Erlenmeyer flask was inverted over the test tube. The test tube and contents were then placed in an oil-bath at 120–130°C. for 24 hours. The level of the solution in the test tube was always kept at the same level as that of

TABLE 1

The decarboxylase method compared with the microbiological method for lysine in the entire series of fifteen proteins.

	AS PERCENTAGES			
	A. In protein (16% N)		B. In whole food	
	Micro-biological	Decarboxylase	Micro-biological	Decarboxylase
Whole egg	8.2	7.8	1.00	0.96
Beef (lean round)	8.1	8.2	1.22	1.23
Horse meat (tough, stringy)	8.5	7.6	1.64	1.47
Halibut (large steaks)	9.7	9.5	1.95	1.90
Haddock (fresh, boneless)	9.4	9.6	1.82	1.86
Soybean flour ¹ (fat extracted)	6.0	5.9	2.98	2.97
Navy beans, dry	6.7	6.4	1.49	1.43
Brewer's yeast ² (blend of three)	6.6	7.4	2.90	3.24
Torula yeast ³	5.7	6.8	2.52	3.00
"Kitchen Food" ⁴ yeast	6.3	6.3	2.78	2.76
Corn germ ⁵ (defatted)	4.8	5.2	1.03	1.10
Wheat germ ⁶ (defatted)	5.7	5.1	2.11	1.89
Cotton seed meal ⁷ (defatted)	4.3	3.4	2.30	1.81
Peanut flour ⁸ (defatted)	3.6	3.0	2.04	1.67
Sunflower seed meal ⁹ (defatted)	3.1	3.0	1.50	1.45

¹ Contributed by The Glidden Co., Chicago, Ill.

² Contributed by The Yeast Products, The Pabst, and The Heffenreffer Companies.

³ *Torulopsis utilis*, through the courtesy of Dr. Thyssen, London, Eng.

⁴ Contributed by the Anheuser-Busch Co., St. Louis, Mo.

⁵ Purchased from The Vio Bin Corp., Monticello, Ill.

⁶ Purchased from The Oil Mill Products Co., Ft. Worth, Tex.

⁷ Obtained through the courtesy of Dr. D. Breese Jones, Washington, D. C.

the oil-bath during hydrolysis. The contents were then transferred to a beaker or shallow dish and dried by warm air or by reduced pressure in a desiccator. This drying was always kept at room temperature.

Hydrolysis for arginine and histidine

Both arginine and histidine analyses as described can be run on diluted HCl hydrolysates of proteins without removing the acid. In these experiments 250 mg of dried defatted protein were placed in a test tube of convenient size. Five ml of 6 N HCl were added, a boiling chip inserted and the tube covered with a glass-bulb air condenser. The mixture was gently refluxed for 24 hours in an oil-bath at 120°C. After hydrolysis the contents of the tube were filtered and diluted to 25 ml. If this solution is again diluted 1 to 10, a sample of 1.0 ml with most proteins will give a color convenient for both the arginine and histidine colorimetric methods of analysis. Neither the final acidity (1.0 ml of 0.12 N HCl) nor the color of the hydrolysate interferes with the Sakaguchi or the Pauly reaction. Adding pure solutions and, alternatively, pure solid arginine and histidine to the food sample before hydrolysis gave recoveries of 95 to 100%.

Hydrolysis for lysine

The destruction of lysine during hydrolysis was carefully checked. When added to 20% HCl and the protein and heated to 120°C. for 24 hours 100% recovery of the amount added was obtained when the hydrolysate was dried down in the desiccator. Although not destroyed by this method of hydrolysis and drying,² a second heating to not higher than 40°C. can be very destructive. Care should be taken therefore in redrying a diluted sample from a hydrolysate, especially from foods with a high lysine content.

² A quick and satisfactory method of drying hydrolysates, not apparently widely known, is the following:

A mixture of flaked sodium hydroxide and calcium chloride (1 to 1) is used as the drying agents in the desiccator. Suction is applied by a water pump and as hydrochloric acid comes off first, the pressure remains high for several hours. When the vapor pressure of water is reached and maintained for an hour the system is closed. If the system is not closed, the drying agents pick up water from the pumps. As the drying agents may become hot, the desiccator must be cooled in a water bath. A sample can be dried by this method in about 36 to 48 hours.

INDIVIDUAL METHODS OF ANALYSIS

Arginine

The determination of this diamino acid in the catholyte, obtained as the second product of electrodialysis for all the basic forms, was carried out by means of the modification of Weber's quantitative use of the Sakaguchi reaction, introduced by Thomas, Ingalls and Luck ('39). The results were conflicting as much because of difficulties with the physical method as with the Sakaguchi reaction. It proved exceedingly

TABLE 2

Comparison of values for "leucine" group by microoxidative and microbiological procedures.

(Percentage figures on basis of 16% N).

	LEUCINE		VALINE		ISOLEUCINE	
	Oxidative	Microbiol.	Oxidative	Microbiol.	Oxidative	Microbiol.
Halibut	11.6	8.8	5.8	6.0	6.1	6.2
Haddock	12.5	8.6	4.2	5.9	3.2	6.2
Beef	13.1	7.7	3.9	5.2	5.5	5.7
Horse meat	10.9	7.8	4.5	5.3	6.5	5.1
Egg	17.8	9.7	5.2	7.2	5.1	7.0
Peanut flour	15.5	6.8	1.3	5.3	4.5	4.6
Cottonseed flour	10.4	6.0	4.6	5.1	4.7	3.9
Sunflower seed	12.0	6.7	3.1	5.6	4.5	5.1
Yeast blend	18.1	7.8	2.7	6.4	6.8	6.0
Wheat germ	8.6	6.6	3.7	6.3	4.0	3.8
Navy beans	11.1	8.2	4.4	6.0	3.6	5.8
Corn germ	10.3	7.7	4.0	5.9	3.5	4.1

troublesome with either of two forms of electrophoretic cells to secure duplicate values for any of the basic amino acids from two aliquots of the dried hydrolysates. Critical examination of the procedures for use of the Sakaguchi reaction also was disappointing because all were found to have serious disadvantages.

As is well known the basic reaction is the coupling of arginine with α -naphthol in alkaline solution, and conversion to a red compound by means of sodium hypobromite. Because excess of the hypobromite destroys the color, attempts

are made to decompose the excess by quickly adding urea solutions, or to run the analysis in the presence of urea. Careful control of the time of adding the reagents and use of varying amounts and concentrations of the hypobromite have been required. Any given procedure in our hands has proved difficult of control so as to obtain a readable color proportional to the concentration of arginine. The required improvement was a means of stabilizing the color. This was accomplished by one of us (G.R.B.) in discovering that *n*-butanol serves not only to extract the red compound from the aqueous mixture but also to stabilize it so that the same reading can be relied upon up to 1 hour after inception.

The reagents are essentially the same as those introduced by Weber ('30). The procedure is as follows: A 5 ml sample of the hydrolysate (or catholyte) containing 5 to 100 μ g of arginine is placed in a test tube, followed by 1.0 ml of 2.5 N NaOH and finally by 1.0 ml of the α -naphthol solution (0.05% in 25% ethanol). Mixing quickly the reaction is completed within 1 minute. Now add 0.2 ml of the hypobromite solution with shaking and immediately blow³ into the mixture 10 ml of *n*-butanol saturated with water. Give the tube a few seconds of vigorous shaking and allow the butanol layer to separate. Suck out the bottom aqueous layer, add 1 ml of 95% ethanol to clarify the butanol solution and read at will up to 1 hour.

The absorption is maximal at 500 $m\mu$. Of course a blank should be run on the reagents which alone give a yellowish color, but with a selective photometer set for clearance at 500 $m\mu$ this color is largely filtered out. It has been found convenient to run the whole reaction in test tubes calibrated to fit the Coleman Universal Spectrophotometer.

Under the conditions described the red color not only is stable but is proportional to the concentration of arginine used. Samples should have a pH and buffering power such that no marked shift is produced by addition of the alkaline

³ Blowing the butanol into the solution with a pipette produces a quicker mixing of the relatively immiscible solvents and a prompter extraction of the color.

reagents. Below pH 11.0 a series of varied color reactions is produced.

All the values given in tables 3 and 4 are derived by this Bartlett modification of the procedures hitherto used.

Histidine

The reagents used are essentially those employed by the MacPherson modification of the Pauly ('04) diazotization reaction. It was confirmed that under the conditions outlined below, low temperature manipulations, a disadvantage of previous modifications of this method, were unnecessary. The amounts of reagents used have been adjusted to get greater sensitivity and convenience for photometric analysis in the Coleman Colorimeter. The whole determination was handled in colorimeter tubes. We found it was unnecessary to wait more than 1 minute for the diazotization-histidine coupling reaction (Macpherson ('42) used 30 minutes).

The procedure is as follows: place 3.0 ml of a sample containing in this volume 2 to 40 mg of histidine in the colorimeter test tube. Add 5 ml of 1% sulfanilic acid solution followed by 0.5 ml of 5% sodium nitrite. After 1 minute the diazotization and the coupling with histidine are complete. Add 1.5 ml of the 20% sodium carbonate to develop the color. Mix well and add 4.5 ml of this 75% ethanol, which substance Macpherson found to be satisfactory for stabilizing the color. The interference of tyrosine in acid protein hydrolysates is negligible.

The color is proportional to the concentration of histidine used, and its maximal absorption occurs at 500 m μ . The whole analysis can be carried out at room temperature.

Adsorption. As an additional check against impurities which might give the Sakaguchi or the Pauly reactions, adsorption separation has been tried. The cation adsorbents Amberlite IR. 100 HAG⁴ and ZeoKarb H⁵ were found to

⁴ Resinous Products and Chemical Co.

⁵ Block and Bolling ('45, p. 42) suggest that a combination of Permutit adsorption (Dubnoff) and Macpherson's method may be the best application of the Sakaguchi reaction.

TABLE 3

Essential amino acids in fifteen food proteins expressed as percentages of $N \times 6.25$ of the acetone-ether insoluble fraction.

	ARGININE	HISTIDINE	ISO-LEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	THREONINE	TRYPTOPHAN	VALINE	% OF A-E INSOL. IN WHOLE FOOD	% N IN A-E FRACT.
1. Whole egg	9.70	3.64	7.00	9.70	7.80	3.88	6.10	4.94	1.63	7.20	15.50	12.72
2. Beef (lean, round)	10.10	4.40	5.70	7.70	8.15	4.12	3.55	5.14	1.46	5.20	16.67	14.46
3. Horse meat												
(tough, stringy)	8.70	4.18	5.10	7.80	7.60	6.23	3.55	4.41	0.61	5.31	22.45	13.80
4. Halibut												
(large steaks)	8.40	3.68	6.20	8.80	9.44	4.48	3.39		1.10	6.00	21.80	14.72
5. Haddock												
(fresh, boneless)	9.20	3.32	6.20	8.60	9.55	4.07	4.14	3.95	1.23	5.90	21.34	14.60
6. Soy bean flour												
(fat extracted)	11.00	3.60	5.70	7.60	5.93	3.59 ¹	5.14	5.24	1.08 ¹	5.40		8.02
7. Navy beans	9.40	3.00	5.80	8.20	6.43	3.88	3.42	3.21	0.49	6.00	84.80	4.21
8. Brewers' yeast												
(blend of three)	13.10	3.00	6.00	7.80	7.40	2.34	3.59	5.07	1.63	6.40	95.70	7.32
9. Torula yeast	8.60	2.80	5.50	8.30	6.84	2.62	4.55	4.97	0.83	5.90	92.50	7.58
10. "Kitchen Food," yeast	7.00	2.84	5.70	6.80	6.34	6.42	4.09	4.88	3.22	5.90	95.80	7.26
11. Corn germ												
(defatted)	10.10	3.60	4.10	7.70	5.17	4.60	2.83	4.22	3.60	5.90	92.60	3.68
12. Wheat germ												
(defatted)	9.60	4.29	3.80	6.60	5.11	2.80	4.08	2.97	2.91	6.30	93.30	6.35
13. Cotton-seed meal												
(defatted)	13.20	3.64	3.90	6.00	3.42	3.49	4.16	3.05	3.86	5.10	88.30	9.60
14. Peanut meal												
(defatted)	13.80	3.00	4.60	6.70	3.01	3.43	2.82	2.61	1.95	5.30	88.60	10.03
15. Sunflower seed meal												
(defatted)	11.30	2.96	5.10	6.80	3.02	3.35	4.96	3.41	2.38	5.60	88.10	8.73

¹% of N of Whole Substance $\times 6.25$.

adsorb the basic amino acids (approximately 100 mg washed adsorbent to 10 ml diluted amino acid solution), but difficulties were encountered in securing quantitative elution (G.R.B.).

The methods for arginine and histidine are satisfactory, however, without this additional check; for they give satisfactory recoveries of the pure amino acids added to the hydrolysates.

Lysine

This one of the basic amino acids has given more trouble than either of the others for securing satisfactory recoveries of known amounts of the pure substance added to the hydrolysates. The assumption that all of the nitrogen remaining in the catholyte after two or three electrodialyses, and precipitation of arginine and histidine, is nitrogen of lysine proved after prolonged trial to be wholly erroneous. Recoveries of pure lysine added to the hydrolysate never were satisfactory. Nor did any of the methods designed for direct application to the hydrolysates (without electrophoretic separation) which were tried prove satisfactory from this standpoint. Consequently resort was had finally to the decarboxylase method of Zittle and Eldred ('44) as confirmed by Neuberger ('45).

This method as modified slightly by one of us (L.E.E.) proved entirely satisfactory for recovery tests. The modification consisted only in running the reaction to completion. The acid tip method as described by Neuberger was used and should give at least 98% recovery.

Recoveries were run at least once, in triplicate or quadruplicate, often twice, on each food analyzed. The method used consisted in the mixing of equal quantities of the hydrolysate and the lysine standard, both containing the same quantity of lysine, as recommended by Dunn et al. ('44). The corrected readings of CO_2 obtained from the mixture on the Warburg manometers should equal one-half the readings obtained from the same volume of each component separately.

Amino acid mixtures without lysine produced no more CO_2 with the decarboxylase than the blanks.

TABLE 4

Essential amino acids in fifteen protein foods expressed as percentages of the whole food.

	ARGININE	HISTIDINE	ISO-LEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	THREONINE	TRYPTOPHANE	VALINE
1. Whole egg	1.19	0.45	0.86	1.19	0.96	0.48	0.86	0.61	0.20	0.89
2. Beef (lean, round)	1.51	0.67	0.86	1.16	1.23	0.72	0.53	0.72	0.22	0.78
3. Horse meat (tough, stringy)	1.68	0.81	0.99	0.91	1.47	1.21	0.69	0.85	0.12	1.03
4. Halibut (large steaks)	1.68	0.74	1.24	1.76	1.90	0.89	0.68		0.22	1.20
5. Haddock (fresh, boneless)	1.80	0.64	1.22	1.67	1.86	0.79	0.81	0.76	0.24	1.14
6. Soy bean flour (fat extracted)	2.75	0.90	1.42	1.90	1.48	1.80	1.28	1.31	0.54	1.35
7. Navy beans	2.10	0.67	1.28	1.83	1.43	0.86	0.76	0.72	0.11	1.34
8. Brewers' yeast (blend of three)	5.70	1.31	2.63	3.42	3.24	1.02	1.50	2.23	0.71	2.80
9. Torula yeast	3.75	1.22	2.41	3.63	3.00	1.15	2.00	2.18	0.36	2.58
10. "Kitchen Food" yeast	3.06	1.23	2.48	2.96	2.76	2.77	1.78	2.12	1.40	2.56
11. Corn germ (defatted)	2.15	0.77	0.87	1.64	1.10	0.98	0.65	0.89	0.77	1.25
12. Wheat germ (defatted)	3.54	1.58	1.41	2.44	1.89	1.03	1.51	1.10	1.03	2.34
13. Cotton-seed meal (defatted)	6.97	1.92	2.06	3.18	1.81	1.84	2.20	1.61	2.20	2.70
14. Peanut meal (defatted)	7.62	1.66	2.55	3.77	1.67	1.90	1.57	1.45	1.10	2.94
15. Sunflower seed meal (defatted)	5.46	1.43	2.78	3.71	1.45	1.61	2.39	1.64	1.14	2.70

Once the sample is freed of excess HCl it must be kept at low temperature (below 30°C.).

This method of determining lysine was checked against the microbiological method described by Stokes et al. ('45) (q.v. below). Table 1 exhibits the results from the two for the protein of the fat-free products and the whole foods as purchased or contributed.

Leucine isoleucine and valine

At the beginning of this work the oxidation method of Fromageot and Heitz ('39) as modified by Block, Bolling and Kondritzer ('40) was employed for determination of these three acids, but results were very variable. Later, even after improvements in technique were made by one of us (R.T.) it was found quite impossible to get satisfactory recoveries of pure substances added to the hydrolysates. The reason may be seen from the following:

By this procedure the branch-chained acids, after conversion to their hydroxy derivatives, yield acetone and the amount from each is readily measured. Since leucine and valine yield acetone but in different quantities depending on the conditions of oxidation, two different oxidative reagents, namely, buffered KMnO_4 and buffered $\text{K}_2\text{Cr}_2\text{O}_7$, are used on each protein hydrolysate. The percentage yield obtained directly on the pure substances with the two oxidants are then combined with the amounts of acetone from the hydrolysates to set up two simultaneous equations expressing the results of each oxidation (see Block and Bolling, '45, p. 224). From these equations the content of leucine and valine in a given hydrolysate may be calculated. Isoleucine gives 2-butanone (ethyl-methyl ketone) and its content is estimated in the same manner by the yields from known quantities of isoleucine. Careful examination of the procedure for leucine and valine, however, revealed that while with oxidation by KMnO_4 the yields of ketones distilling over from pure leucine and valine did not change when each was added to amino acid mixtures or to hydrolysates, with oxidation by $\text{K}_2\text{Cr}_2\text{O}_7$ the yields found

after addition of the pure acids to mixtures or to protein hydrolysates definitely decreased from the values obtained on the pure branch-chained acids. Such results would be expected to cause the calculated values for leucine to be too high and those for valine to be too low (i.e., with yields as found in this laboratory which differed always in the direction illustrated by the example; after KMnO_4 leucine 24%, valine 65%; after $\text{K}_2\text{Cr}_2\text{O}_7$, leucine 10%, valine 50%).

To avoid such discrepancies another method for these acids was sought and the microbiological method of Stokes and associates ('45) came to our attention.⁶ Satisfactory control curves, close agreement between duplicate samples and good recoveries of the pure amino acids were experienced using the organism *Streptococcus faecalis*, as advocated by the authors.

A comparison of the values found for the three acids by the two methods—the micro-oxidative and micro-biological—may be seen for a dozen proteins in table 2. It is clear that the oxidative procedure gives a consistently higher content of leucine and a consistently lower content of valine than does the microbiological, just as predicted for calculated and true values from long experience by one of us (R.T.) with the micro-oxidative procedure. The percentages for isoleucine determined oxidatively fall on either side of, or coincide with, the values determined microbiologically.

The percentages for the three acids found for the fifteen food proteins and the corresponding whole foods reported in tables 3 and 4, respectively, are those obtained by the microbiological method.

Methionine

The determinations were made by the micro-chemical colorimetric method of McCarthy and Sullivan ('41) without modification. Pure crystalline methionine⁷ added to the hydrolysates gave average recoveries of 97%.

⁶ Through the good offices of Dr. C. D. Kochakian, Dr. Stokes very kindly furnished a description of their procedure previous to its publication.

⁷ Merck.

Phenylalanine

This amino acid was determined by Block's ('38) adaptation of the Kapeller-Adler ('32) method and checked later by Block and Bolling's ('45, p. 109) adaptation of the Kapeller-Adler-Kuhn procedure (Block and Bolling, '45, p. 108). Recoveries of the pure amino acid added to the hydrolysates averaged 100% (range 87 to 109%).

Threonine

This determination was made by the periodate method of Shinn and Nicolet ('41) without modification. Average recovery of pure threonine was 98% (range 94 to 103%).

Tryptophane

The alkaline hydrolysis especially devised for determination of this amino acid was carried out as follows. The dried acetone-ether insoluble fraction showing negligible loss of nitrogen in the reagents was used. With fairly pure proteins like egg and the meats 70 mg samples were sufficient, but with the vegetables containing residues of roughage and starch larger samples were required. The appropriate amount of the dry material was weighed into 20 mm strong Pyrex test tubes and 2 ml of 5 N NaOH added per 70 mg of dry substance. The test tubes were then sealed with an oxygen-gas flame and heated at 115-116°C. for 16 to 18 hours in an oil-bath. In the autoclave 6 hours at 15 lbs pressure are sufficient. Some of the materials thus sealed up caused the tubes to explode. They were mostly materials of the vegetable type, but the horse meat used was particularly explosive. In these cases larger, or heavier-walled tubes had to be used. In the autoclave of course explosion is prevented by the outer pressure.

Tryptophane in samples hydrolyzed in the manner described was found to be stable for 24 hours. Check experiments, in which both 100% and 50% of the amount contained in the hydrolysate (determined colorimetrically) were added to the

dried material before hydrolysis, showed average recoveries of 99% and 98%, respectively.

The alkaline hydrolysate was neutralized with 20 N H_2SO_4 to a pH of 7.0.

The colorimetric method of Lugg ('37, '38) with the modification suggested by Brand and Kassell ('39) was used. However, in running blanks it was found that dilution of the hydrolysate with water gave results that were too variable, so the blank used was an exact duplicate of the unknown. Also in the final step 0.5 ml of water was used instead of the sodium nitrite. Otherwise the combined Lugg-Brand and Kassell procedure was unchanged. Average of recovery in the colorimetric determination was 95%.

SUMMARY AND CONCLUSIONS

The methods used, and controls employed for their verification, in the analyses of fifteen high protein foods for the ten essential amino acids are described. Several of the methods available in the literature at the time this work was undertaken (September, 1943) after thorough trial, had to be discarded as inadequate. This was notably true of the electrophoretic method for separation of the three basic amino acids as well as the current colorimetric procedure for these acids singly and the oxidative method for determination of isoleucine, leucine and valine. Criticisms of these methods are given.

Of the final results presented in table 3, those which apply to corn germ and beefsteak represent closely the values used in paper III of this series for making up the mixtures of the essential amino acids in imitation of these two proteins. The principal discrepancies concerned leucine, which was nearly equally too high (4-5%) and valine equally too low (1.3%) in both foods. The biological values of the mixtures in these two instances, after correction for the unnatural isomers, were brought close to those of the proteins they imitated.

All methods which could be controlled by recoveries of the pure amino acids were so controlled and no procedures were

adopted which did not give average recoveries of 95% or better.

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BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN

V. COMPARISON OF THE AVERAGE EFFECT OF TEN SINGLE AMINO ACIDS WITH EXTRA EGG PROTEIN AS SUPPLEMENTS TO AN EGG DIET ¹

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INTRODUCTION

Ever since the discovery by Weiss and Rapport ('24) that the specific dynamic effect of alanine and glycine is completely neutralized by feeding them together with casein or gelatin, it has been a problem to know whether this is a general phenomenon or one which occurs only with certain amino acids and proteins of a peculiar composition. We have not been able to find any confirmation of their findings from other combinations.

The most plausible explanation of this suppression of a thermogenic effect, among several which the authors suggest, it would seem, is the combination of alanine and glycine into peptides which are more readily retained than those formed from the protein alone. For if alanine or glycine were simply mixed with other free amino acids and so absorbed, unless

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester.

there were polypeptide formation on the way to the liver (a very short journey), there should be more total amino acids brought under the deaminating power of the liver in a given time after feeding and therefore (probably) greater thermogenesis. In other words there would be a summation of the specific thermogenesis of amino acid with that of the protein.

Now if it be supposed that non essential amino acids can form such peptides with the products of proteolysis, so that they escape deamination (which presumably must precede the heat effect) how much more reasonable is it to suppose that essential ones would escape this fate, under such circumstances. Particularly would this seem to be a conservative measure if the supplemented protein were poor in essential amino acids. On the other hand supplementation of high biological value proteins with essential amino acids presumably would not be so likely to enhance retention, for that would be "to carry owls to Athens."

The specific thermogenic effects are secondary to our present interest. Work is in progress in the laboratory on this property of the essentials and will be reported upon in due time. However, the disclosure as to which if any of this group added as supplements to a high biological value protein, at a level of intake insufficient to establish nitrogen equilibrium, can or cannot enhance gross retention, or, still better, that more refined order of retention which is called biological value, would point the way to rewarding studies of thermogenic effects. Little is known of this aspect of amino acid metabolism. Is it all governed by what the tissues need or does the resistance to oxidative deamination or to transamination, operating in a purely chemical manner, play a part in the fate of a supplement? Schoenheimer and associates ('38, '40) have shown with isotopes N^{15} and deuterium that one of the essential amino acids, lysine, does not participate in the exchange of amino groups except as donor and once deaminated cannot be reaminated. This would appear to presage a good thermogenic effect: but Doty and Eaton ('37) already had shown that lysine has no specific dynamic effect in the dog.

Perhaps we may find that certain amino acids out of the group of essentials, precisely because they cannot readily become re-aminated and so be isolated from tissues as such, nevertheless can readily be combined into peptides and consequently have reserved for them the more conservative and, shall we say more "noble" role of promoting synthesis. This kind of inquiry might well lead to a rating of the amino acids in a biological-value scale of their own.

It was natural for this laboratory to choose egg protein for such a study both because of its established superiority and because of the experience with it gained here from previous studies. Whole egg protein fed at a level of 5% of the calories alternatively with other proteins enables a diet squad, with other fundamental requirements met, to maintain a good nutritive condition for considerable periods of time. At this level it provides a good base of reference for relative biological values and at the endogenous level, or lower, it furnishes a suitable liason for the interconvertibility of biological values to the absolute scale (Murlin, Edwards, Hawley and Clark, '46b). The present problem required a level of intake lower than that necessary for nitrogen equilibrium.

The diet squad for this group of experiments was the first one organized for the program described by the general title of the series. It consisted of six men and four women ranging in age from 20 to 36 years, in weight from 45 to 83 kg and in height from 156 to 176 cm. They were selected from the undergraduate, graduate and medical students of the University and included one teaching and research assistant. All were in sound health at the beginning of the series, and, with the exception of three who fell victims of a prevailing epidemic of influenza and consequently were incapacitated during the last two periods and two others who partially succumbed to the epidemic for a day or two, all remained well and fit. There were some distressing symptoms from ingestion of two of the amino acids in particular, dl leucine and dl methionine, but these disappeared promptly when ingestion ceased (see table 3 for footnotes concerning these reactions).

PLAN AND PROCEDURE

Besides attempting to establish differences in retention potency, not to dignify the term too much, among the ten essential amino acids there was another objective; namely, to compare the combined "potency" of the several acids with that of a quantity of egg protein, likewise superimposed on the basal egg diet and supplying to all members simultaneously approximately the same quantity of essential nitrogen as the average supplied to all the ten subjects taking one amino acid each as supplement. Should the squad be able to tolerate as single amino acids the same quantity of nitrogen, say 1 gm, as the egg supplement contained, and continue so to do for an equal length of time, we should have what might be called a vertical measure as well as a horizontal one, of the effect on B.V. This would be the more convincing if two experiments with the single amino acids should give average results which agreed well. Furthermore, and this was the reason for beginning the general inquiry as we did, it would not be necessary to wait for completion of the analytical work on the egg protein. The experiment would be a test as to whether the whole of the nitrogen of the racemic forms or only that of the natural isomers should be used in such a comparison, when the correct analysis became available.

After a preliminary adjustment period of 4 days, the distribution of calories was fixed at protein 4, fat 45 and carbohydrate 51%, the total calories at 48 cal./kg of net body weight, and collections of excretion were started. In period II the egg nitrogen was continued at the same level, making this the definitive pre-experimental control period. In period III the egg nitrogen was increased for each person by exactly 1 gm, allowance being made in the carbohydrate for the 25 extra cal. from protein.

Period IV, the post-experimental control had to be at the same time the pre-experimental control for the first period of amino acid supplements because of time limitations. For this reason and in order to insure good retention of the amino acids it was desirable to start them from a state of assured

minus nitrogen balance. The basic intake level therefore was reduced in period IV by 0.31 gm N average, below that ingested in period II. The consequence was that the difference between the ingestion of the two control periods averaged together and that of the experimental period was increased to 1.16 gm N daily instead of the 1.0 gm originally intended. This made the nitrogen of the natural isomers of essential amino acids in the egg supplement as shown by later analyses 0.7 gm average daily (table 1) instead of the postulated 0.5 gm.

For the amino acid supplements this same level of basic egg protein was maintained through periods V to IX. The calorie distribution and the total calories also remained the same for each person except for very minor adjustments. Because of difficulty in tolerating certain of the amino acids experienced by four different subjects, the average nitrogen from all ten of the essentials actually ingested daily was 0.768 gm for the first experiment and 0.77 gm for the second (table 3) instead of 0.8 gm which would have been the average for both, if all had gone well. The average actual total nitrogen from natural isomers was 0.46 gm in both experiments.

METHODS

In the diet kitchen weighed samples of all foods, approximating the average amount ingested by the squad members daily, were assembled in tightly sealed jars and kept in the refrigerator. The eggs always were as fresh as could possibly be purchased under war restrictions and were delivered from the farm directly to the hospital. They were freshly broken, homogenized and kept stirring while being sampled for each person's allowance at the different meals.

All items of the diet were regularly analyzed for nitrogen content, samples being taken from the homogenized material, either while the Waring, or other type of blender, was in motion; or, for repeats, after thorough mixing. The macro Kjeldahl method was more suitable for these food products than the micro methods and therefore was used for the excreta. Feces were separated into periods by ingestion of a

capsule of carmine taken immediately before the first breakfast of each new period. In this particular series aliquot samples by weight were taken from each stool while fresh and the aliquots for each subject assembled in a jar of adequate size for use of the Waring blender. In later series it was found that each subject's feces could be collected for the entire period in a 1-gallon "candy jar" placed directly in the commode. The jar contained a 5% solution by weight of nitrogen-free H_2SO_4 with a trace of copper sulfate dissolved in it to precipitate the H_2S . A special rotary motor provided with paddles constructed after the Waring design but more resistant to the acid was used to homogenize the stools at the end of each period. The contents always were freshly stirred for sampling.

Once started, continuous nitrogen balances were recorded for every subject.

RESULTS

From our best analysis (Edwards, Sealock, Bartlett et al., '46) for the ten essential amino acids in whole egg, repeated in table 1, may be calculated the average amount of each contained in the control periods II and IV as compared with the total of each in period III when the extra egg was ingested.

It appears that in the experimental period each person ingested 58 gm extra whole egg substance containing 4.47 gm of the ten essentials. All the other constituents of the diet remained unchanged. The essentials made up 61.6% and the non essentials 38.4% of the 7.25 gm total protein represented by the 1.16 gm nitrogen.

In table 2 are seen the condensed data concerned in the effect of this egg supplement on the individual nitrogen balances for the three periods. The gross additional nitrogen retentions, calculated for each person on the basis of his own extra protein ingestion, were found to be very variable — from 38 to 98%. It is of interest to note that the average ingestion of 1.10 gm extra nitrogen (an increase of 28%) by four women produced an average improvement in nitrogen balance (gross retention) of 0.945 gm or 86% of the extra ingestion;

TABLE 1

Average amounts of the ten essential amino acids contained in the whole egg substance fed in periods II and IV compared with period III.

	IN WHOLE EGG	AV. IN PERIODS II AND IV	IN PERIOD III	DIFFERENCE III - (II + IV) 2
	gm/100 gm	gm/206 gm	gm/224 gm	
Arginine	1.19	2.45	3.14	0.69
Histidine	0.45	0.92	1.18	0.26
Isoleucine	0.86	1.77	2.27	0.50
Leucine	1.19	2.45	3.14	0.69
Lysine	0.96	1.98	2.54	0.56
Methionine	0.48	0.99	1.27	0.28
Phenylalanine	0.86	1.77	2.27	0.50
Threonine	0.61	1.26	1.61	0.35
Tryptophano	0.20	0.41	0.53	0.12
Valine	0.89	1.83	2.25	0.52
Total	7.69	15.83	20.30	4.47 = 0.7 gm N

Average extra natural isomers ingested in period III, 4.47 gm.

Average extra whole egg nitrogen ingested in per III, 1.16 gm.

TABLE 2

Effect of 1.16 gm extra egg N on the nitrogen balances, average daily (gm).

SUBJ. AND SEX	CONTROL II (6) ²	EXP. III (5)	CONTROL IV (6)	AV OF II AND IV	GAIN OF III	% OF EXTRA N RET ¹
1 ♂	-0.035	+0.497	-0.166	-0.100	+0.597	54
2 ♀	+0.501	+1.181	-0.354	+0.073	+1.108	98
3 ♂	+0.551	+0.627	-0.200	+0.175	+0.452	38
4 ♀	+0.469	+1.158	-0.224	+0.122	+1.036	92
6 ♀	+0.599	+0.958	-0.297	+0.151	+0.807	80
7 ♂	+0.066	+0.226	-0.701	-0.317	+0.543	48
8 ♂	-0.084	+0.145	-1.192	-0.638	+0.783	67
9 ♂	-0.823	+0.336	-0.884	-0.853	+1.189	89
10 ♀	-0.434	+0.312	-0.602	-0.518	+0.830	74
11 ♂	+0.158	+0.801	-0.248	-0.045	+0.846	71
		+0.624		-0.195	+0.819	
				Average		71%
				Average 4 F's		86%
				Average 6 M's		61%

¹ Calculated on individual differences between N fed in periods II and IV (average) and that fed in period III. This difference (av. for all 10 subjects) was 1.159 gm. It varied from 1.004 gm for subj. 6 to 1.334 gm for subj. 9.

² Figures in () indicate no. of days in the periods.

while the average extra ingestion of 1.2 gm (26%) by six men produced an improvement of only 0.730 gm or 61% of extra nitrogen ingested. Only 2% difference in the extra ingestion made a difference of 25% in gross retention. It appears that women are better conservators of nitrogen. The average for the whole group without regard to sex is 0.819 gm or 71% of 1.16 gm extra nitrogen.

Taking the basic egg protein as having a biological value of 100% and rating the augmented amount in period III against this base, it is found that the biological value is 93.1% — a reduction of 7%. But the difference between the sexes is not so great as for percentages gross retention: viz. females 93.3, males 90.6 (table 4). Of course from the nature of the calculations it could not be so great.

The average extra amount of whole egg substance (58 gm) is not far from the amount supplied in one large White Leghorn egg. The basal level is represented approximately by four medium sized eggs. Hence if the average person were subsisting on four eggs a day as his sole source of protein in a diet containing adequate calories, the addition of one large hen's egg would assuredly put him well over the equilibrium line: for he would retain some 70% of the extra egg protein (0.8 gm N) and this would provide him with a composite of the ten essentials weighing 4.47 gm, and making his total supply for the day 20.3 gm (table 1). We must not overlook the fact that 38.4% of the egg protein consists of the non essential amino acids, and this experiment obviously throws no light on which of them is retained. Supplementary egg protein, however, is not so efficiently utilized for maintenance of live tissue or for deposit as reserve protein, as the case may be, as is the submaintenance supply represented by the basal amount; hence the lower B.V.

Single essential amino acids as supplements

These experiments were carried out in the same manner as the single one above, i.e., the periods were arranged in the same order (IV, V and VI making one cycle and VI, VII and

IX constituting a second, each with the experimental embraced between two controls). But instead of administering the same supplement to all members of the squad simultaneously they were each given a single and separate amino acid (one of the ten to each of the ten), in an amount calculated to supply 0.5 gm N from the natural isomer alone. All the dl forms except tryptophane supplied 1.0 gm N and all the others 0.5 gm, tryptophane included because, the evidence available at this time indicated that both l and d isomers of this essential were nearly equally well utilized (retained) by man.

In table 3 are presented the essential data for calculation of the percentages of the actual amino acid nitrogen ingested which were retained in the two experiments. Considering the vicissitudes which the squad members suffered from the distasteful qualities of some of the amino acids and from influenza (see footnotes) it is surprising how nearly equal the average figures are for amounts ingested and retained² in the two experiments. The credit is due to the pluck and persistence of the squad members and the competence of the assistants in the dietetic and analytical work. The hope for an agreement between these two experiments was realized adequately to form some basis of opinion as to the comparative benefits one may expect from natural and artificial supplements supplying essential amino acids.

The average retention, both in weight and as percentage of the amount of nitrogen ingested, is as nearly equal in the two experiments as could be expected. The average improvement in nitrogen balance compared with the average improvement from the egg supplement (71% of ingested) is not disappointing when we remember that egg supplies nothing but natural isomers whereas the average nitrogen from natural

²It should be stated that the calculation for percentage of amino acid nitrogen retained in each instance was based on the full 8 days of each period concerned, except for subjects P.F. and A.L. in period V of the first experiment and subjects J.A. and P.M. in period VII of the second experiment for whom the calculations included only the first 2 full days and any additional days for which correction could be made. Reasons for these omissions from the periods named are fully explained in the footnotes to the table.

isomers supplied by the synthetic amino acids is only 4.46 gm or 60% of the total. When the retention, or improvement in nitrogen balance, is calculated on this basis the percentage is 91 and 93% for the two experiments, respectively. Clearly it is the natural isomer in the dl forms one must count on to match the effects on nitrogen balance from comparable amounts of nitrogen in natural foodstuffs (see paper III, Murlin, Edwards, Fried and Szymanski, '46a).

It is evident from table 3 that the three basic amino acids produced high retentions in both experiments; that isoleucine and leucine produced one low and one high in each — the high by the woman of the pair in both and low by the man; that phenylalanine and valine produced moderate retentions, each yielding a value above 50% and each yielding one in the neighborhood of 20 to 30%, the man of each pair scoring the higher figure; and finally, that methionine, tryptophane and threonine yielded retentions so low as to suggest that the egg protein already contained all of these three that the body could utilize to advantage.

For the performance of the two sexes we should include only the eight amino acids that were used as supplements by both. Thus it is found that the eight tests on women produce the average retention of 84%, the eight on men 42% — just half as much. The difference is greater than with egg protein as the supplement. This striking sex difference can scarcely be explained by greater tolerance by the women; for the evidence, though not a little complicated by influenza symptoms, is the other way. It seems more probable that the men may either metabolize the amino acids more rapidly by having a greater potential for oxidative deamination or that they may excrete them unchanged more rapidly (see below under biological values).

Biological values

There are three points of interest in this effect of the amino acid supplements: (1) the comparison of the two experiments; (2) comparison of the sexes and (3) the effect of the amino

TABLE 3
Summary of N ingestion and N retention from each essential amino acid added as supplement to egg diet.

BOTH EXPERIMENTS	FIRST EXPERIMENT, PERIODS IV, V, VI				SECOND EXPERIMENT, PERIODS VI, VII, IX			
AMINO ACIDS	Subject and sex	gm N ingested	gm N retained	% re-tained	Subject and sex	gm N ingested	gm N retained	% re-tained
<i>l</i> (+) arginine	T.L. ♂	0.50	0.377	75	M.G. ♀	0.50	0.628	125
<i>l</i> (+) histidine	M.M. ♂	0.50	0.518	103	J.S. ♂ ¹	0.50	0.539	108
<i>l</i> (+) lysine	M.G. ♀	0.50	0.921	184	T.L. ♂	0.50	0.738	147
<i>dl</i> isoleucine	E.L. ♂	1.00	0.330	33	P.F. ♀ ²	1.00	1.057	106
<i>dl</i> leucine	P.F. ♀	0.89 ³	1.597	180	E.L. ♂	1.00	0.190	20
<i>dl</i> methionine	A.L. ♂	0.79 ³	—0.242	—30	J.A. ♂	0.77 ⁴	0.138	18
<i>dl</i> tryptophane	J.S. ♂	0.50	—0.060	—12	P.M. ♀	0.43 ⁵	0.101	23
<i>dl</i> phenylalanine	P.M. ♀	1.00	0.208	21	A.L. ♂	1.00	0.524	52
<i>dl</i> threonine	V.V. ♀	1.00	—0.002	0	M.M. ♂	1.00	0.030 ⁶	3
<i>dl</i> valine	J.A. ♂	1.00	0.539	54	V.V. ♀ ⁷	1.00	0.313	31
Average		0.768	0.419	54.5		0.77	0.426	55.4

¹ This subject had nausea headache, heart palpitation, abdominal cramps Took amino acid only 3 days — correction for amt. not taken third day.

² Subject had nausea, weakness, dizziness. Correction for amt. not taken third day (the last).

³ These three subjects absent from second control period IX.

⁴ Subject had nausea, dizziness, weakness. Continued to 4½ days, but omitted part on fourth day. Correction for this.

⁵ Symptoms of influenza; also nausea and vomiting. Correction for amt. lost 1 day.

⁶ Subject confined to bed with influenza last 2 days of period VI. Average used for first 4 days.

acids on the fecal nitrogen excretion. To discuss these intelligently requires a display of all the data for evaluation of biological values, as given in table 4.

The average figures at the end of the table show that the effect of these several supplements taken together on "biological value"³ of the egg protein is an experiment which can be readily duplicated. However, there is one criticism that can fairly be raised; namely, the absence of three members of the squad from the second control period IX. Study of the original nitrogen balance data fails to reveal any feature of this period which disqualifies it for standing as a control for the remaining seven members, notwithstanding that the intervening period VIII⁴ postponed the control 6 days; for they were in precisely the same average state of balance for undertaking the second control as if they had started it 6 days earlier. It is practically certain that the same would have been true of all ten if it had been possible for them to continue to the end. The only consideration remaining then, is whether period VI alone as control would give the same average re-

³It will be understood that this is not a true or absolute biological value in the conventional sense but an effect on the percentage of the absorbed which is retained, compared with egg unsupplemented, in other words, a relative B.V.

⁴Period VIII represented an attempt to profit by the high retentions shown in the first experiment (and indicated by early analysis of the urine in the second) by feeding all members the five amino acids which had exhibited retentions higher than 50% and to furnish all the supplement from these alone in combination. The mixture contained: 3.76 gm l (+) arginine; 28.69 gm l (+) lysine, 14.97 gm l (+) histidine—all as the monohydrochlorides—also 18.74 gm dl leucine and 16.74 gm dl valine, calculated to supply 1.2 gm total nitrogen to each of eight subjects remaining at that time, and so proportioned as to provide 1.0 gm nitrogen from natural isomers, from the five, in the order named as follows: 0.1, 0.4, 0.3, 0.1, and 0.1 gm. This mixed supplement to the same egg diet as used in periods VI and IX was continued for 3 days, when the leucine was dropped (because of some symptoms ascribed to it) and the remaining four amino acids were readjusted to supply 1.1 total nitrogen per person, containing 1.0 gm N from natural isomers in the following proportions: arginine 0.2, lysine 0.4, histidine 0.3 and valine 0.1 gm.

The average retentions from the seven members who completed this period and period IX calculated against the nitrogen balances of the same periods VI and IX as controls was 43% from the mixture of five and 35.3% from the mixture of four amino acids. Evaluated on the basis of nitrogen of natural isomers only these values became 52 and 42%, respectively.

sults for percentage retention and biological value for the remaining seven as do the controls VI and IX. If so it is clear that the inclusion of the results for the three members having only one control is valid. The answer is that the average nitrogen retention and the average B.V. would have been some 4% higher and 2.8% (points of B.V.) lower, respectively, than those averages for the seven (not shown in table) now are. In other words the present average retention for the second experiment (table 3) is probably 1.3% too high and that for average B.V. (table 4) is about 1.1% too low (remembering that B.V. is always a percentage). These obviously are not significant errors incurred by using results for the three subjects who had only one control period.

The difference between the average biological values for men and women, 90.6 ± 7.06 and 93.3 ± 10.14 , respectively, is too small to be statistically significant as is evident from the standard deviations and the small number of observations. It would not require more than twice the number, with no greater variation, to produce a difference between means which would be significant. The reasons for such an outcome judging by results already obtained, would be: (1) That women have a greater average correction on the fecal nitrogens, as will be evident in casting the eye down column C of table 4; while (2) men have a greater average correction on the urinary nitrogens, as seen in column I. The former being negative increases the nitrogen absorbed and thereby tends to decrease B.V., but the difference is slight; while the larger correction on the urine, being positive has a greater effect to reduce the amount of nitrogen retained by men and therefore to reduce B.V. still more. These effects are evident from the averages (at the bottom of the table) for the two groups of eight tests on men and eight on women taking the same amino acids.

Effect of amino acids on fecal nitrogen

In fifteen out of twenty comparisons in table 4 of the fecal nitrogens from the unsupplemented egg diet and the supple-

TABLE 4

Biological value of egg protein and individual amino acid supplements in terms of egg alone.

AMINO ACID SUPPLEMENT AND SUBJECT	CONTR. PERIOD	EXPL. PERIOD	CONTE. PERIOD	A A.A. FECAL N	B EGG ALONE FECAL N	C A-B	D EGG AND A.A. FECAL N	E ABSORBED N D-O	F TRUE DIGEST. %	G EGG AND A.A. URINE N	H EGG ALONE URINE N	I G-H	J $\frac{E}{I \times 100}$	K B. V. (100-J)	SEX
Valine J.A.	V	IV	VI	1.112	1.301	-0.189	5.217	5.406	104	3.804	3.181	0.623	11.5	88.5	♂
Valine V.V.	VII	VI		0.674	0.550	+0.124	4.695	4.571	97	3.588	3.025	0.563	12.3	87.7	♀
Leucine P.V.	V	IV	VI	0.740	0.866	-0.126	5.232	5.358	102	3.470	4.050	-0.580	-10.8	110.8	♀
Leucine E.L.	VII	VI	IX	1.578	1.187	+0.391	5.493	5.102	93	4.051	3.638	0.413	8.1	91.9	♂
Methionine A.L.	V	IV	VI	0.699	1.068	-0.369	5.538	5.907	107	5.083	3.675	1.408		76.2	♂
Methionine J.A.	VII	VI	IX	1.273	1.408	-0.135	4.991	5.126	103	3.944	3.163	0.781	15.2	84.8	♂
Lysine M.G.	V	IV	VI	0.604	0.904	-0.300	4.851	5.151	106	3.459	3.580	-0.121	-2.3	102.3	♀
Lysine T.L.	VII	VI	IX	0.855	0.938	-0.083	4.318	4.401	102	3.256	3.441	-0.185	-4.2	104.2	♂
Arginine T.L.	V	IV	VI	0.854	0.979	-0.025	4.319	4.344	101	3.816	3.697	0.119	3.4	96.6	♂

Histidine J.S.	VII	VI		0.739	0.735	+0.004	5.006	5.002	100	4.503	4.548	-0.043	-0.8	100.8	♂
Isoleucine E.L.	V	IV	VI	1.231	1.280	-0.049	5.493	5.542	101	4.484	3.764	0.720	13.0	87.0	♂
Isoleucine P.F.	VII	VI		0.746	0.740	+0.006	5.342	5.336	100	4.315	4.396	-0.081	-1.5	101.5	♀
Phenylalanine P.M.	V	IV	VI	0.609	0.669	-0.060	4.220	4.280	101	3.737	2.885	0.852	20.0	80.0	♀
Phenylalanine A.L.	VII	VI	IX	0.749	1.050	-0.301	5.745	6.045	105	4.461	3.684	0.777	12.8	87.2	♂
Tryptophano J.S.	V	IV	VI	1.196	0.922	+0.274	5.006	4.732	94	4.810	4.524	0.286	6.0	94.0	♂
Tryptophane P.M.	VII	VI	IX	0.634	0.683	-0.049	3.647	3.696	101	3.257	2.877	0.380	10.3	89.7	♀
Threonine V.Y.	V	IV	VI	0.550	0.593	-0.043	4.693	4.738	101	4.199	3.154	1.049	22.1	77.9	♀
Threonine M.M.	VII	VI	IX	0.698	0.815	-0.117	5.875	5.992	102	5.452	3.974	1.478	24.7	75.3	♂
Averages				0.847	0.919	-0.072	4.976	5.048	101.4	4.072	3.638	0.434	8.4	91.6	
Average of minus values						-0.149			Average for Exp. 1					91.2	
Averages for the tests on 8 men and 8 women taking the same amino acids.				♀ 0.640	0.738	-0.098	4.691	4.789	102	3.718	3.436	0.282	6.7	93.3	
				♂ 1.046	1.059	-0.014	5.183	5.197	100	4.267	3.722	0.534	9.4	90.6	

¹ Only one control period on account of influenza.

mented one the nitrogen from the latter is less than that from the former, notwithstanding that for the entire 3 to 6 days of ingestion this diet contained anywhere from 0.43 to 1.0 gm more nitrogen daily. Does this mean that the presence of these amino acids directly stimulates additional absorption of egg protein over the amounts absorbed in the fore and after periods? Or does it mean that when the free amino acid is taken in considerable quantity (from 1.88 gm arginine to 11.8 gm phenylalanine) every day it acts either before absorption or after it enters the blood to increase secretion from the alimentary glands thereby reducing the amount of food nitrogen excreted? Preliminary experiments⁵ in this laboratory indicated strongly that single amino acids dissolved in salt solution and infused slowly into the vein of an anaesthetised dog enhanced quite definitely after a short latent period the action of secretion, injected a short time before, to increase pancreatic secretion. There was also in a few experiments a somewhat reduced content of trypsin in the secretion collected during the augmented flow. This latter observation is in accord with those of Grossman, Greengard and Ivy ('43) on the trypsin content of rat pancreases resulting from the substitution of hydrolyzed casein⁶ for casein in the rat's diet for 21 days. Increased flow of pancreatic juice, if continued for only 6 days might however go on without change of concentration of the enzyme, or produce proportionally less decrease in concentration, and so, because of better mixture with the alimentary contents, result in more complete digestion of the egg protein, sufficient to reduce the nitrogen content of the feces to the extent noted.

SUMMARY AND CONCLUSIONS

A comparison was made of the average effect on nitrogen balance of feeding to each of ten members of a diet squad a single but different one of the ten essential amino acids, with the effect of a small amount of whole egg protein added as a

⁵ Performed by D. W. Kramer, C. J. Scarpellino and E. K. Ryder under direction of J. R. M.

⁶ Amigen.

supplement to the same diet containing already an inadequate amount of this protein. The average total nitrogen from the amino acids fed singly as supplements should equal the nitrogen of the egg supplement. Actually owing to some vicissitudes experienced in taking some of the amino acids and the exigencies of time this ideal was not realized very exactly. Two experiments on the amino acids with different persons taking each one gave good agreement and furnished what may be called a vertical average in comparison with a horizontal (simultaneous) one on the egg supplement.

Another objective was to learn something of the relative retention potency of the several essentials and whether the total nitrogen of the racemic forms should be reckoned against the egg nitrogen or only that of the natural isomers.

The results were: (1) That the supplement of egg (learned from analyses obtained later) supplied 0.7 gm essential amino acid nitrogen of natural form, while the ten synthetic essentials supplied on the average only 0.46 gm from natural isomers. (2) That the average retention of the egg nitrogen was 0.819 gm or 71% of the total nitrogen fed, indicating that some non essential nitrogen must have been retained; while the average retention from the ten essentials was, in two experiments, 0.419 and 0.426 gm or 54.5 and 55.4%, respectively, of the average total nitrogen fed in the essentials, indicating a large wastage of nitrogen from the amino acids. (3) Calculated on the basis of the nitrogen from the natural isomers only the retentions in the two experiments were 91 and 93%, respectively. (4) The effects on biological value relative to the egg basal diet were about the same from the two types of supplement; namely a reduction of seven points in the percentage scale.

Other observations were that women appear to be better conservators of nitrogen than men, and that in fifteen of twenty tests the presence of amino acids in the diet reduced the fecal nitrogen, notwithstanding that each subject ingested from 0.43 to 1.0 gm more nitrogen than from the basal egg diet alone.

Conclusions reached were: (1) That a vertical average can be duplicated satisfactorily and furnishes much useful information, but would be still more useful after a correct analysis of the protein was in hand. (2) That the individual essential amino acids differ greatly in retention potency under the conditions of these experiments; and (3) that it is the nitrogen of the natural isomers which must be counted on to equal the effects on nitrogen balance of natural proteins.

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REPRODUCTION AND LACTATION STUDIES WITH RATS FED NATURAL RATIONS¹

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In studies of the dietary insufficiency of a corn-soybean ration for swine, Ross ('43), Ross et al. ('44), Cunha ('44), and Cunha et al. ('44) found that a ration composed of 76.35% yellow corn, 17.50% soybean oil meal, 5.0% alfalfa meal, and 1.15% minerals failed to support normal reproduction and lactation. Normal reproduction and improved lactation resulted, however, when the basal ration was supplemented with 10% additional alfalfa meal. Certain vitamins, commercial preparations, and other feeds were shown to be ineffective supplements. These workers concluded that good quality alfalfa meal carried a factor or factors which wholly, or in part, corrected the deficiencies of the basal ration. It was also demonstrated that the ration which sows received during growth markedly influenced subsequent performance in reproduction and lactation.

Ross ('43) and Cunha ('44) report that similar corn-soybean oil meal-alfalfa meal rations did not support optimum growth in the rat. The addition of all the ten known B-complex vitamins did not stimulate growth to any appreciable

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extent. Cunha ('44) states that the possibility of a vitamin imbalance, or of some effect on intestinal synthesis or absorption, should not be overlooked, but suggests that some unidentified factor or factors were still needed to adequately supplement the basal ration for growth. Alfalfa, acid-washed casein plus choline, tankage, brewer's yeast, or 1:20 liver powder supplemented the basal ration. Alcohol-washed casein plus choline, vitamins fed singly or in combination, and certain other natural materials were ineffective supplements. Very little work has been done on studying the reproduction of the rat on a natural ration made up entirely of concentrates of plant origin.

We have studied reproduction and lactation in rats fed similar corn-soybean oil meal-alfalfa meal rations. An assay for determining supplements to the basal ration which would promote normal reproduction and lactation has been developed. Certain feeds and purified materials have been tested. The results of these experiments are reported in this paper.

EXPERIMENTAL

Females of Sprague-Dawley breeding were used. In some experiments the females were started on their rations at weaning, but in other experiments they were fed as indicated. Females were mated at 12 or 14 weeks of age. Males from the stock colony were used and were replaced each week by new stock colony males. All groups of females were kept in wire cages and fed ad libitum. The pregnant females were removed from the larger colony cages to smaller individual wire cages without false bottoms. Wood shavings were used for nesting. Two days after parturition all litters were reduced to six young. The length of the lactation period was 21 days and all litters reaching this age were weaned.

The basal ration was composed of ground yellow corn 75.34%, soybean oil meal (expeller process) 17.50%, alfalfa meal 5.0%, CaHPO_4 1.0%, CaCO_3 0.65%, NaCl (iodized) 0.50% and MnSO_4 0.01%. Two drops halibut liver oil were given weekly by dropper. All major supplements to the basal

ration were made at the expense of the corn. Rations were mixed every 2 weeks and were stored at room temperature except in the case of high fat, or high mineral rations. These latter rations were stored in the dark at refrigerator temperatures.

RESULTS

Experiment I

The first experiment was designed to study the effect of the basal ration on reproduction and lactation in the rat. All females were fed the basal ration during growth and reproduction. Results are summarized in table 1.

TABLE 1

The effect of the basal ration on reproduction and lactation.

Total number females	19
Number sterile	7
Resorptions and toxemic deaths	4
Number litters born	8
Number live young	33
Number dead young ¹	6
Number young given to females to raise ..	27
Number young weaned	0

¹ Only those young found with the female are tabulated. Many were known to have been eaten by the female.

A large percentage of the females fed the basal ration were sterile. A peculiar type of hemorrhage was produced in the fetal attachments of the pregnant rats fed such a ration and resulted in expulsion of the dead fetus at term, or death of the fetus, attempted resorption, or toxemia and death of the female. Four of the females fed the basal ration died during or shortly after parturition. The breakdown of the capillaries of the cotyledons in many cases occurred during the early part of the gestation period as indicated by a bloody discharge by the pregnant female. In this case when the capillary breakdown occurred early, the rat was able to resorb the developing embryos. However, if this condition occurred a day or two before parturition, the female was not able to resorb the

dead embryos and as a result gave birth to dead young, or died from toxemia. Although these symptoms are similar to those described for a vitamin E deficiency, α -tocopherol supplementation (4 mg daily) was without effect. Young that were born alive appeared normal at birth, but did not live more than 1 or 2 days following parturition. Although the young attempted to nurse, no milk could be found in their stomachs. When this condition was observed in these experiments it was considered to be due to lactation failure.

Experiment II

Experiment II was designed to study the effect of previous dietary treatment upon reproduction and lactation in the rat. All females received the basal + 10% alfalfa meal ration during attempted reproduction and lactation. Rations fed prior to this period are indicated in table 2 where results are summarized.

TABLE 2

Effect of previous ration on reproduction and lactation.

LOT	RATION FED PREVIOUS TO ATTEMPTED REPRODUCTION	RATION FED DURING ATTEMPTED REPRODUCTION	TOTAL NO. FEMALES	NO. LITTERS BORN	NO. LIVE YOUNG	PER- CENTAGE OF YOUNG WEANED
1	Basal ration (birth to breeding)	Basal + 10% alfalfa	11	8	50	0
2	Basal ration (weaning to breeding)	Basal + 10% alfalfa	8	7	39	25
3	Basal + 10% alfalfa (second generation)	Basal + 10% alfalfa	9	8	22	71
4	Basal + 10% alfalfa (weaning to breeding)	Basal + 10% alfalfa	17	17	86	86

Complete failure in lactation resulted in lot 1 when the females were raised from birth to breeding on the basal ration. The females of lot 2 were fed the basal ration from weaning to reproduction. This group weaned 25% of the young which were born alive. The females of lot 3 were fed only the basal + 10% alfalfa meal ration and were raised from females

that received a similar ration during reproduction and lactation. The females of lot 4 received only the basal + 10% alfalfa meal ration but were raised from females that received a stock ration² during reproduction and lactation. The second generation performance of females on the basal + 10% alfalfa meal ration (lot 3) was not as good as the first generation performance (lot 4). In the latter group, 86% of the young were weaned. The basal plus 10% alfalfa meal ration was in this case a borderline carrier of the factors needed to supplement the basal ration. These data indicate that the factors needed for reproduction and lactation can be stored by the female rat.

Experiment III

The effect of adding practical supplements to the basal ration was studied next. Females were raised until 10 weeks of age on a stock ration.² At this time they were placed on the basal ration for a 2-4 week "depletion period." At the end of the "depletion period" the females were placed on experimental rations. After a 2-week period, they were mated. Using this procedure it was possible to study the effects of adding some natural supplements to the basal ration. Results are summarized in table 3.

Reproduction on the basal ration was again very poor but somewhat better than had been obtained in experiment I. The addition of 10% alfalfa meal to the basal ration resulted in marked improvement in reproduction. Forty litters were born from forty-nine females, and the number of resorptions and sterile animals was reduced when 10% alfalfa meal was added. Brewer's yeast, tankage, soybean lecithin, a combination of casein (crude or acid-washed) plus choline, and 1:20 liver powder when added to the basal ration, also caused a reduction in the number of resorptions and the number of sterile females.

²Stock ration composed of ground yellow corn 70.0%, linseed oil meal 14.0%, casein 5.0%, alfalfa 4.0%, liver 2.0%, butter 3.0%, CaCO₃ 1.0% and NaCl (iodized) 1.0%.

Lactation performance was very poor on the basal ration; only 3% of the young given to the females to raise were weaned. The addition of 10% alfalfa meal or of 5.0% 1:20 liver powder markedly increased the percentage of young weaned. Results using a limited number of animals suggest that fish meal, soybean lecithin or a combination of casein (crude or acid-washed) plus choline furnished a factor or factors which also improved lactation performance.

TABLE 3
Effect of natural materials on reproduction and lactation.

BASAL RATION SUPPLEMENT	TOTAL NUMBER FEMALES	RESORP- TIONS	NUMBER LITTERS BORN	NUMBER YOUNG GIVEN TO FEMALES TO RAISE	NUMBER YOUNG WEANED	PER CENT YOUNG GIVEN TO FEMALES THAT WERE WEANED
Basal ration only	65	19	35 ¹	115	3	3
Alfalfa meal 10.0%	49	1	40	158	80	51
Brewer's yeast 5.0%	11	0	10	38	12	32
Tankage 5.0-10.0%	25	1	20	99	24	24
Fish meal 5.0%	7	1	4	21	21	100
Fish "press H ₂ O" 5.0%	4	1	2	11	3	27
Wheat 75.35%	9	1	8	46	3	7
Soybean lecithin 0.3%	4	0	4 ¹	7	5	71
Casein (crude or acid-washed 5.0%) + cholino (0.3%)	11	2	8	36	22	61
Casein (alcohol extracted, 5.0%) + choline (0.3%)	8	4	2	12	3	25
1: 20 liver powder 5.0%	22	2	16	74	51	69

¹ Three litters born dead.

Wheat and a combination of alcohol-extracted casein + choline were ineffective supplements. Other materials not included in table 3 that were tested and shown to be ineffective supplements include wheat bran, wheat middlings, dehydrated oat grass, wheat germ, wheat germ oil and wheat germ meal. Six to ten females were used to test each of these materials not included in table 3.

DISCUSSION

Very little work has been reported on reproduction and lactation of rats fed all plant rations.

Various experiments with rats have shown that cystine and methionine effectively supplement certain soybean rations (Hayward et al., '36; Evans and McGinnis, '46). Other studies have shown that sulfur-containing amino acids promote lactation in rats when fed rations in which the protein is derived from alfalfa leaf meal (Wright and Haag, '39).

Alfalfa meal has been shown by Ross et al. ('44) and Cunha et al. ('44) to aid reproduction and lactation in swine fed rations similar to the basal ration used in the studies reported in this paper. Fairbanks et al. ('45) have reported that the addition of 10% alfalfa meal to certain natural rations containing adequate amounts of nutrients known to be required by the pig, resulted in effective gains and decreased death losses in young pigs.

The addition of certain natural and purified materials to the basal corn-soybean oil meal-alfalfa meal rations markedly improved reproduction and lactation. Alfalfa meal and 1:20 liver powder were effective supplements. Limited numbers of animals suggested that certain other materials, including combinations of casein (acid-washed or crude) plus choline, favorably improved the reproduction and lactation performance. Alcohol-washed casein, a relatively pure protein, plus choline was found to be an ineffective supplement. Further experiments are now being conducted to determine whether the factor or factors necessary to supplement the basal ration are of a known or an unknown nature.

The possibility of a vitamin imbalance or of some effect on intestinal synthesis or absorption should not be overlooked. Experiments have indicated that certain dietary factors not required in the diet when purified diets are fed, may be required when rats are fed certain soybean rations (Cunha et al., '43; Spitzer and Phillips, '46). Patton et al. ('46) suggest that sardine fish meal contains a factor which will stimulate growth in chicks fed a corn-soybean diet and that the need for this factor may be a peculiarity of the corn-soybean diet.

Results of experiment II show a marked difference in reproduction and lactation depending upon past dietary history of the females. This is also indicated when experiments I and III are compared. Although the basal females in both of the latter experiments failed to lactate, reproduction was somewhat better in experiment III where the rats had received a stock diet during growth. Thus it was demonstrated that a storage of dietary essentials needed for reproduction and lactation takes place with the rat. These results are in agreement with swine experiments of Ross ('43), Ross et al. ('44), Cunha ('44), Cunha et al. ('44). This suggests that it is important for one to know the previous ration fed the animals and to evaluate results obtained accordingly.

SUMMARY AND CONCLUSIONS

It has been shown that an all plant ration composed of 75.34% yellow corn, 17.50% soybean oil meal, 5.0% alfalfa meal and 2.16% minerals was inadequate for reproduction and lactation in the rat. More than 35% of the females fed the basal ration were completely sterile. Resorption and toxemia occurred frequently. Young that were born alive died within 1 or 2 days after parturition. These young attempted to nurse, but no milk could be found in their stomachs. This condition was considered to be due to lactation failure.

Previous diets were found to have a pronounced influence upon reproduction and lactation performance. There appears to be a storage of dietary essentials needed for reproduction and lactation in the rat.

The supplementation of additional alfalfa meal, 1:20 liver powder, a combination of casein (crude or acid-washed) plus choline, or fish meal improved reproduction and lactation. These data indicate that an active factor or factors are present in these supplements. Brewer's yeast, fish press water, soybean lecithin, among other materials, were variable in their supplemental effect upon reproduction and lactation.

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THE INFLUENCE OF CALORIC INTAKE ON THE GROWTH UTILIZATION OF DIETARY PROTEIN¹

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FOUR FIGURES

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The protein efficiency method of Osborne, Mendel and Ferry ('19) has been, with modifications, one of the most extensively employed methods for the evaluation of the nutritive quality of proteins. The suggestion was made by Bosshardt et al. ('46b) that differences in the reported nutritive indices of a protein by different workers may be due, at least in part, to differences in experimental technique. The effect on the protein efficiency ratio of differences in the pre-test standardization of the test animals, the duration of the test period, and the level of the test protein in the diet also were shown.

In the adult animal a restriction of the caloric intake results in an increase in urinary nitrogen excretion, indicating a decrease in the utilization of protein nitrogen. This has been related to the sparing action of carbohydrate on urinary nitrogen excretion (Lusk, '28). The data of Allison, Anderson and Seeley ('46) indicate that fat as well as carbohydrate is effective in decreasing the urinary nitrogen excretion.

¹ A preliminary report of this data was presented before the American Institute of Nutrition, Atlantic City, March 12, 1946.

It has been reported (Barnes et al., '45) that a restriction of protein intake resulting from the feeding of a diet containing a small percentage of protein or by a restriction of the entire diet through paired-feeding causes a decrease in the growth utilization of protein. It is probable that the major factor causing a decreased protein utilization under these conditions is the level of protein consumed. However, another factor that is common to both of the above conditions and may be involved is the decreased caloric intake.

The possibility exists that in protein evaluation studies involving ad libitum feeding differences between proteins may be exaggerated because of variations in the ill-defined and often overlooked appetite aspects of the diets, which result in marked differences in total food intake and thus in caloric intake. The increased growth utilization of casein when liver fractions were added to the diets, which was reported by Bosshardt et al. ('46a), may have been due, at least in part, to an increased food intake resulting from the correction of an unrecognized dietary deficiency.

This study was designed to investigate the influence of caloric intake on the growth utilization of proteins by rats and mice.

EXPERIMENTAL METHOD

Groups of eight male albino weanling rats (Sprague-Dawley strain), in individual cages, were fed isocaloric diets containing varying amounts of extracted whole egg ad libitum for 42-day periods (Barnes et al., '45). Determinations were made of weight gains and of food and protein consumptions. The typical protein utilization curve that is shown in figure 1 was obtained. The average daily energy intake per cm^2 of body surface area was calculated² for each group and also is shown plotted as a function of protein intake in figure 1.

In a second experiment, groups of seven male albino weanling mice (Sharp and Dohme, Swiss-Webster strain), in individual cages, were fed isocaloric diets containing varying levels of casein for 10-day periods (Bosshardt et al., '46b).

² B.S. = $11.36 \times \text{wt.}^{\frac{2}{3}}$ (Carman and Mitchell, '26).

Weight gain and food and protein intake were measured. The protein utilization and energy intake data are shown in figure 2.

In both cases, at the level of maximal protein utilization there was a maximal consumption of total calories per unit body surface area. The caloric intake per unit of body surface area decreased on either side of this point. This pattern of caloric intake and protein utilization also has been ob-

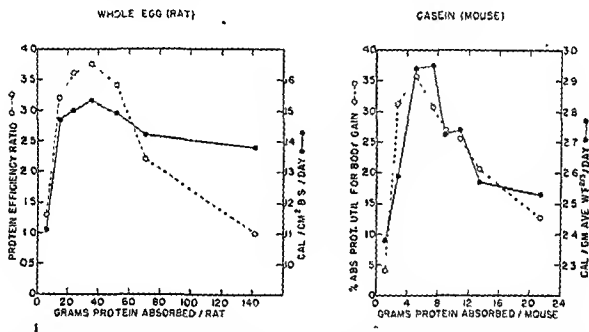


Fig. 1 The relationship between protein utilization and caloric intake with varying protein intakes. Diets containing varying levels of whole egg protein were fed to rats for 42-day periods.

Fig. 2 The relationship between protein utilization and caloric intake with varying protein intakes. Diets containing varying levels of casein were fed to mice for 10-day periods.

tained with rats that were fed isocaloric diets containing different levels of wheat gluten and two different soyflours, and with mice that were fed isocaloric diets containing different levels of extracted whole egg and wheat gluten.

It cannot be concluded from these data that optimal calories are supplied when protein utilization is maximal. However, it would appear possible that, at any level of protein intake, the growth utilization of protein may be enhanced if the non-protein caloric intake is increased.

As a preliminary experiment to determine the influence of the caloric intake on the growth utilization of protein, five diets with varying caloric content were employed. The basic diet composition was the same as previously reported (Bosshardt et al., '46b) and contained 10% casein, but the fat:dextrin ratios were altered so that the various diets contained from 2 to 32% fat with a corresponding range of caloric values of 3.9 Cal. per gm to 5.5 Cal. per gm. These diets were fed to male weanling albino mice (Sharp and Dohme, Swiss-Webster strain), in individual cages, for 10-day periods. Weight gain and food and protein intake were recorded. The results obtained are shown in table 1.

TABLE 1

Protein efficiency ratios determined with mice in 10-day feeding periods with diets of varying caloric contents but all containing 10% casein.

FAT IN DIET	CAL./GM DIET	PROT. EFF. RATIO	N IN CARCASSES
%			%
2	3.9	1.75	2.77
12	4.4	1.77	2.88
22	5.0	2.09	2.79
27	5.2	2.20	2.72
32	5.5	2.29	2.68

As the fat level in the diet was increased with a corresponding increase in the caloric value per gm, there was a marked increase in the protein efficiency ratios which ranged from 1.75 to 2.29. However, as the fat level was increased the food intake per mouse for the 10-day period decreased from 24.8 gm to 17.4 gm with a corresponding decrease in protein intake from 2.3 gm to 1.7 gm. The total caloric intake per mouse per day, however, increased from 9.8 to 11.3. The carcass nitrogen figures indicate that in no case was there an abnormal fat deposition that would give misleading protein efficiency ratios.

Results of this nature are difficult to evaluate because of the marked differences in protein and caloric intake, neither of

which were the same for any two groups. This may help to explain some of the differences in reported nutritive indices of the same protein.

Two series of studies to investigate the influence of caloric intake on the growth utilization of protein under conditions of ad libitum feeding, but with essentially equal protein intake were set up.

Three series of diets containing a well-heated soyflour as the protein source were prepared. All diets contained 4% salt (Wesson, '32) and to each 100 gm of diet were added 0.4 mg of riboflavin, 0.4 mg of thiamine hydrochloride, 0.4 mg of pyridoxine hydrochloride, 2.5 mg of niacin, 1.1 mg of calcium pantothenate, 7.5 mg of para-aminobenzoic acid, 200 mg of choline hydrochloride, 20 mg of inositol and 0.05 mg of 2-methyl-1,4-naphthoquinone diacetate. The water soluble vitamins were mixed with a sucrose carrier and alpha tocopherol (2.5 mg) was dissolved in the fat. In addition, each animal received 2 drops of a cod liver oil twice weekly. The remainder of the diets consisted of a well heated soyflour, hydrogenated cottonseed oil,³ sucrose, and fiber.⁴ In the first series of diets the soyflour was incorporated at such a level that the protein accounted for approximately 10% of the total calories, and the fat, fiber, and sugar were varied to give a range of 2.5 to 6.6 Cal. per gm of diet. In the second series the soyflour was added so that all diets contained 10% protein by weight and the fat, fiber, and sugar were varied to give diets with a range of 2.6 to 6.5 Cal. per gm. The third series consisted of isocaloric diets with protein levels ranging from 8.1 to 13.9% by weight, or 7.7 to 13.5% of the total calories.

The different diets were fed ad libitum to groups of eight male weanling albino rats (Sprague-Dawley strain), in individual cages, for a period of 42 days. A record was kept of weight gain and of food and protein intake. From the data were selected those groups in which the average protein intake

³ Crisco.

⁴ Cellu flour.

per animal corresponded to that giving maximal utilization (Barnes et al., '45).

In figure 3 is shown the relationship between protein utilization, calculated as the protein efficiency ratio, and the average daily caloric intake per cm^2 of body surface area per day. These data indicate that there is a limit to which additional non-protein calories can enhance the growth utilization of protein. When, however, the caloric intake is reduced to below 80% of the maximal intake obtained in this experiment there is a very marked reduction in the protein utilization.

A similar experiment using mice as the test animals and casein as the protein source was carried out. The basal diet consisted of 2% corn oil,⁵ 20% glucose,⁶ 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellulose,⁴ 1% Wilson's 1:20 liver concentrate powder, and was supplemented so that each 100 gm of diet contained 4 mg of alpha tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1,4-naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 200 mg of choline chloride, and 21.6 mg of inositol. The remainder of the diets consisted of casein and hydrogenated cottonseed oil⁷ the levels of which were varied so as to give three series of diets similar to those previously described.

The diets were fed ad libitum to groups of seven male weanling albino mice (Sharp and Dohme, Swiss-Webster strain) for 10-day periods. Determinations were made of weight gain and of food and protein consumption. Again the data were selected to include only those groups whose protein intakes corresponded to that giving maximal utilization (Bosshardt et al., '46b). The results are shown in figure 4.

As in the previous study with rats a limit was approached above which the utilization of protein could not be increased

⁵ Mazola.

⁶ Cerelease.

⁷ Primex.

by increasing the intake of calories. In this experiment a sharp reduction in protein utilization occurred when the caloric intake was below 95% that of the maximal intake obtained.

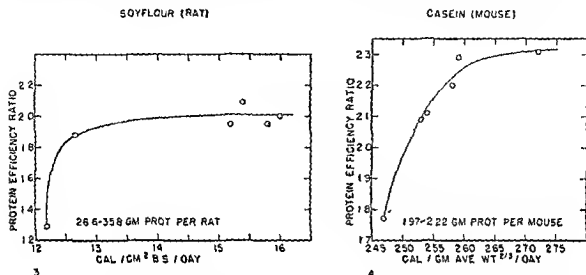


Fig. 3 The relationship between protein utilization and caloric intake with approximately equal protein intakes (see text). Diets containing a well-heated soyflour as the protein source were fed to rats for 42-day periods.

Fig. 4 The relationship between protein utilization and caloric intake with approximately equal protein intakes (see text). Diets containing casein as the protein source were fed to mice for 10-day periods.

DISCUSSION

Larson and Chaikoff ('37) have indicated that, under proper conditions, the feeding of additional carbohydrate to adult dogs that were considered to be in a "normal" state of nutrition reduced the urinary nitrogen excretion, indicating a sparing effect of carbohydrate on protein metabolism. The diet maintaining the "normal" state of nutrition supplied 60 Cal. per kg of body weight per day. Melnick and Cowgill ('37) and Allison and Anderson ('45) have shown that the minimal protein intake required to maintain nitrogen balance in adult dogs varied with individual dogs. These results were obtained by providing all test animals with an equal caloric intake. Allison and Anderson ('45) observed in one group of three dogs that an increase from 80 to 100 Cal. per kg of body weight per day resulted in a decreased urinary nitrogen excretion with a corresponding decrease in the amount of protein necessary to maintain nitrogen balance.

Allison, Anderson and Seeley ('46) have defined the nitrogen balance index as the rate of change of nitrogen balance with respect to absorbed nitrogen. The nitrogen balance index is equal to the biological value of Thomas ('09) (the proportion of absorbed nitrogen retained by the body) if it can be assumed that nitrogen excretion on a protein-free diet is "endogenous" nitrogen. The data of Allison and Anderson ('45) indicate that within limits a variation of the non-protein caloric intake alters the urinary nitrogen excretion without a change in the nitrogen balance index. If the feeding of additional non-protein calories decreases the urinary nitrogen excretion without altering the nitrogen balance index, the effect must be one of decreasing the "endogenous" nitrogen metabolism of the animal.

More recent results (Allison, Anderson and Seeley, '46) have indicated that the nitrogen balance index in adult dogs is not affected until the caloric intake is reduced to below 50% of that considered by them to be adequate for optimal protein utilization. A reduction of the caloric intake to 25% of that adequate for optimal protein utilization resulted in a marked decrease in the nitrogen balance index. At no level of protein intake was positive nitrogen balance obtained at the 25% caloric intake level.

The results of Allison and co-workers would suggest that when the non-protein caloric intake is reduced systematically two types of response are encountered. In the first type, which is observed when the caloric restriction is relatively small, the nitrogen balance index remains essentially constant, although the "endogenous" metabolism changes. In the second type, where a more severe caloric restriction is imposed, there is a marked decrease in the nitrogen balance index.

The data obtained in the present study may be considered to correspond to the first type of response since the animals were in positive nitrogen balance and changes in caloric intake were small. As is shown in figures 3 and 4, with essentially constant protein consumption decreases in caloric intake resulted in decreases in protein efficiency ratios (gm gain in

body weight per gm of protein consumed). There was, however, a range of caloric intake that maintained maximal protein utilization. Increasing the calorie consumption beyond this was without effect on the protein efficiency ratio.

In the growing animal an increase in "endogenous" excretion will result in a smaller proportion of the absorbed protein that is used in new tissue formation. This in turn will result in a decreased protein efficiency ratio. However, since growing animals are in positive nitrogen balance, the decrease noted in the protein efficiency ratio when the caloric intake is decreased most probably is not a reflection of a decrease in the nitrogen balance index but rather of an increase in "endogenous" metabolism.

These studies have made possible the estimation of caloric intake per unit body size that is necessary for the optimal growth utilization of soyflour protein and casein by rats and mice, respectively. There is no evidence that these values can be applied to other dietary proteins. However, it is of considerable importance to point out that with poor proteins such as wheat gluten supplied in ordinary isocaloric diets, caloric intake never reaches the plateau levels shown in figures 3 and 4 regardless of the amount of protein in the diet. This may mean that voluntary food intake is so severely inhibited by poor proteins that suboptimal caloric intake results. It is well recognized that poor proteins give lower nutritive value indices in growing animals than in adult maintenance studies (Barnes et al., '46). Part of the discrepancy between the two types of evaluation may be due to self-imposed caloric deficiencies that cause an exaggerated decrease in the utilization of the low quality proteins.

It has been pointed out previously (Bosshardt et al., '46a, '46b) that various alterations in experimental technique may cause differences in the nutritive indices of proteins. This report indicates the necessity of the control of the non-protein caloric intake if optimal values are to be obtained. Diets may be compounded in such a manner that, even though the animals are eating *ad libitum*, a self-imposed caloric restriction that

is sufficient to result in suboptimal protein utilization may occur.

SUMMARY

Caloric intake and protein utilization have been compared in growing rats and mice that were eating ad libitum.

With each protein source it was found that the level of intake exhibiting maximal protein utilization coincided with a maximal caloric intake per unit of body surface area.

At a given level of protein intake, changes in caloric consumption often resulted in changes in the apparent utilization of protein.

It is important in determinations of protein quality to maintain the non-protein caloric intake at a level at which optimal values will be obtained.

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protein to be converted to carbohydrate and the addition of more carbohydrate should not lower the S.D.A. of protein very greatly. On the other hand, when an animal with an R.Q. of 0.72, as in Ring's work, is given protein, this is probably converted in considerable part to carbohydrate with a large waste of energy. Carbohydrate given with protein should reduce this tendency. Furthermore, fat given to a rat with a low R.Q. and burning much fat already would probably not effectively reduce the S.D.A. of protein.

The question which arises from these observations is, "Does a diet high in fat or one high in carbohydrate have the lower S.D.A.?" According to Ring's observations, the S.D.A. of a diet high in fat should be slightly greater than that of one high in carbohydrate. On the other hand, Forbes and Swift state, "While there is a question as to the applicability of results obtained with one species to the nutrition of another species, the results of this study suggest, at face value, that it is not necessary to diminish the protein content of the hot weather human diet in order to have a low heat increment, since this purpose can be accomplished by equicaloric substitution of fat for carbohydrate.

"It thus appears that there is a physiological economy in the high fat content of the diet of working people of some tropical countries — notably Brazil."

METHOD

In order to test these opinions, we have prepared two diets each with the same amount of protein, minerals and vitamins. One diet was high in fat and the other high in carbohydrate. The composition of each is shown in table 1.

During a previous study, we had been using small rats (about 50 gm) and in order to reduce control measurements we have continued this practice. These rats were kept on one of the above diets for at least 1 week prior to the first metabolic measurements. The Haldane principle was used for determining the gaseous metabolism, and protein was assumed to be burned in the proportion ingested. The respira-

tion chambers were kept at 30°C. The control measurements were made over a period of 7 hours during which no food was allowed. The next day the rats ate a weighed amount of food, and the metabolism was subsequently determined, this time for a period of 8 hours. Only quiet periods were used in calculations though it was found that the figures for S.D.A. were almost the same if one subtracted the total metabolism of the control period from the total after the ingestion of food. In other words, the activity of the rats was about the same whether fed or not.

TABLE 1
Composition of diets.

	HIGH CARBOHYDRATE DIET	HIGH FAT DIET
Casein (vitamin free) — gm	214	214
Corn oil — gm	40	261
Cod liver oil — gm	20	20
Sucrose — gm	626	118.5
Yeast (autoclaved) — gm	60	60
Salt mixture no. 1 (U.S.P. XII p. 637) — gm	40	40
Succinylsulfathiazole — gm	10	10
Thiamine ¹ — mg	10	10
Ca pantothenate ¹ — mg	20	20
Choline ¹ — mg	1,000	1,000
Inositol ¹ — mg	1,000	1,000

¹ Kindly supplied by Merck and Co., Inc., Rahway, N. J.

It is possible that 8 hours is not long enough to obtain the entire S.D.A. This, however, is not important for these results since the S.D.A. of a high fat diet should last longer than that for a high carbohydrate diet, and the true value of S.D.A. for the fat will, if anything, be more reduced than that for carbohydrate. Thus the evidence will be weighed in favor of a low S.D.A. for the fat diet.

RESULTS

In table 2 are shown the S.D.A.s of animals maintained on high fat or high carbohydrate diets. The S.D.A.s appear to be

about the same for each group. Though the mean figures show the S.D.A. for the fat diet ($7.10 \pm 0.60\%$) to be higher than that for the carbohydrate diet ($6.65 \pm 0.33\%$), the standard deviations indicate that the true mean for the fat diet may lie very slightly below that for carbohydrate.

The intake of food just prior to these measurements of S.D.A. was kept approximately the same for each group—23.1 Cal. per 100 gm of rat for those receiving the carbohy-

TABLE 2

The respiratory quotients and the specific dynamic effect of a high carbohydrate and a high fat diet.

HIGH CARBOHYDRATE DIET			HIGH FAT DIET		
Respiratory quotients		S.D.A. ¹	Respiratory quotients		S.D.A. ¹
Fasting	Fed		Fasting	Fed	
(0.640)	0.914	3.47	0.753	0.730	5.98
0.715	0.862	7.71	0.724	0.768	14.18
0.804	0.983	5.87	0.754	0.779	6.21
0.756	0.910	6.56	0.722	0.713	3.97
0.827	0.941	4.76	0.743	0.733	6.35
0.787	0.863	8.91	0.731	0.722	7.70
	0.960	6.11	0.709	0.748	4.93
0.846	0.950	8.41	0.831	0.723	8.78
0.965	1.044	6.52	0.716	0.733	6.93
0.787	0.969	8.92	0.752	0.742	6.01
0.846	0.849	6.27			
0.803	0.883	8.44			
0.777	0.936	4.56			
Av. 0.810	0.928	6.65	0.743	0.739	7.10
± 0.015	± 0.010	± 0.33	± 0.007	± 0.004	± 0.60

¹ Calculated as per cent of calories in food consumed.

drate diet, and 22.0 Cal. for those on the fat diet. The slightly smaller food intake on the fat diet will tend to keep the S.D.A. low on this regime, since Forbes, Kriss and Miller ('34) have shown that the S.D.A. increases with increase in food intake.

The measurement of metabolism on the high fat and high carbohydrate diets gave quantitatively the same results both during control determination and after feeding when calcu-

lated per 100 gm of rat per hour. For the high carbohydrate feeding, the control and "after feeding" values, respectively, were 0.812 Cal. and 0.998 Cal.; for the high fat feeding, the corresponding values were 0.810 and 1.002 Cal.

The respiratory quotients were, of course, high with carbohydrate and low with fat (see table 2).

If the S.D.A. for these two types of diet were markedly different, it might well be reflected in food intake. The food ingested by groups of four animals has been carefully measured over a period of 2 or 3 weeks. Subtracting from these results the figures for the additional energy stored in the bodies of these growing rats (see Wynn and Haldi, '44), we obtained the following results, given in Cal. per 100 gm per day: for high carbohydrate diet 26.79, 28.19, 31.45 and 32.20, with an average of 29.66; for high fat diet 29.77, 30.70, 31.27 and 31.99, with an average of 30.93.

These figures do not include any correction for loss of energy in the feces or urine. They do include the energy required for normal activity. The observations suggest that as much energy is required when ingesting a high fat diet as when living on a high carbohydrate regime. All the above observations are in accord with previous work of Ring ('42). They show that when fat is substituted for carbohydrate, the S.D.A. of the diet is not reduced (see Forbes and Swift, '44).

CONCLUSIONS

1. The S.D.A. on a high carbohydrate diet ($6.65 \pm 0.33\%$ of energy in food ingested) is about the same as that of a high fat diet ($7.10 - 0.60\%$) (see table 2) containing the same amount of protein.

2. Respiratory metabolism for quiet periods during the 8 hours subsequent to the ingestion of a meal high in carbohydrate averaged 0.998 Cal. per 100 gm per hour, and is almost the same as after ingesting an isocaloric meal high in fat — 1.002 Cal. per 100 gm per hour.

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EFFECT OF PROTEINS LOW IN TRYPTOPHANE ON GROWTH OF CHICKENS AND ON LAYING HENS RECEIVING NICOTINIC ACID-LOW RATIONS ¹

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ONE FIGURE

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That young chickens require a dietary source of nicotinic acid, when fed certain purified diets, was demonstrated by Briggs, Mills, Elvehjem and Hart ('42). Briggs ('45) reported that the addition of 10% of gelatin to a highly purified ration caused an increase in the nicotinic acid requirement of chicks and that tryptophane acted similarly to nicotinic acid in preventing the deficiency.

Previously, it had been found by Krehl, Teply, Sarma and Elvehjem ('45), working with rats, that the inclusion of tryptophane or nicotinic acid in certain low casein diets prevented a deficiency produced by the feeding of corn. Recent reports by Krehl, Sarma, Teply and Elvehjem ('46) and Krehl, Sarma and Elvehjem ('46) showed that the growth-

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inhibiting effect of corn was related to the nature of its protein and that similar inhibiting effects could be produced with certain non-corn rations.

In addition to results secured with the chicken and rat, Chick, Macrae, Martin and Martin ('38) and Wintrobe, Stein, Follis and Humphreys ('45) in studies with the pig, and Axelrod, Morgan and Lepkovsky ('45) with the dog, have also noted a relationship between the protein content of the ration and the nicotinic acid requirement. The relationship of the tryptophane content of the protein to the nicotinic acid requirement of these animals was not established in these cases.

Studies with various animals have established the fact that the presence of high amounts of corn, or products derived from corn, in a diet may increase the nicotinic acid requirement. In work with chickens, Sarma and Elvehjem ('46) reported that the addition of large amounts of corn grits to a nicotinic acid-low ration had a deleterious effect on growth, which was counteracted by the feeding of nicotinic acid. Scott, Singesen and Matterson ('46) also reported that chicks receiving a simplified diet containing corn and gelatin required nicotinic acid to prevent perosis and for growth. In work with rats, Krehl, Teply and Elvehjem ('45a) produced a nicotinic acid deficiency by feeding high amounts of corn. These results with the rat have been confirmed by Dann ('46a). Handler ('43) suggested that the presence of corn meal in rations for dogs may be a causative factor in pellagra. Krehl, Teply and Elvehjem ('45b) reported that when corn grits were added to a synthetic ration, the nicotinic acid requirement of the dog was increased. In work with swine, Davis, Freeman and Madsen ('40) noted necrotic enteritis, due to a deficiency of nicotinic acid, especially prevalent in pigs fed diets rich in corn in contrast with other grains. It has been known for a number of years that pellagra in humans was associated with large amounts of corn or corn products in the diet. The work with humans has been reviewed (Anonymous, '45).

The results presented in this paper extend the former observations on the relationship of proteins low in tryptophane

to the nicotinic acid requirement and, in addition, offer evidence for the need of nicotinic acid in the diet of laying hens. Studies with a practical ration are also given. A convenient method of assay with the chick for the nature of the inhibitory action of protein is presented.

EXPERIMENTAL

Day-old New Hampshire chicks, of mixed sexes, were divided into uniform groups of six and raised in electrically heated batteries with wire floors. Feed and water were given ad libitum. Weighings and other observations were made weekly and the experiments conducted for a period of 4 weeks.

The basal ration, 108GN, low in nicotinic acid and low in a source of arginine and glycine, consisted (in %) of Cerelese 68.4, crude casein 18, Liver Fraction L 3, soybean oil 4, Salts 1M (Briggs, '46) 6, l-cystine 0.3. Each 100 gm also contained the following amounts of vitamins (in mg): thiamine HCl 0.4, riboflavin 0.8, Ca pantothenate 2.0, choline HCl 200, pyridoxine HCl 0.6, biotin 0.02, i-inositol 100, p-aminobenzoic acid 0.2, 2-methyl-1,4-naphthoquinone 0.1, and alpha-tocopherol 0.5. Vitamin A and vitamin D₃ (1200 U.S.P. units and 170 A.O.A.C. units, respectively) were fed by dropper weekly. This ration is similar to that used by Briggs et al. ('42) which contained 0.3 mg of nicotinic acid per 100 gm. Substitutions in the ration were made at the expense of the carbohydrate.

Ration 2 was designed to be similar to certain practical-type rations (containing no animal protein supplement) and also to be fairly low in nicotinic acid and high in corn. It was composed as follows: ground yellow corn 50, ground oats 10, wheat middlings 5, wheat bran 5, soybean oil meal 13, corn gluten meal 13, oyster shell flour 2, steamed bone meal 1.4, sodium chloride 0.5, manganese sulfate 0.012, vitamin D₃ (Delsterol) 200 A.O.A.C. units per 100 gm, and riboflavin 0.4 mg per 100 gm. Substitutions to this ration were made at the expense of corn. Ration 2 was calculated to contain approximately 4.5 mg of nicotinic acid per 100 gm by use of the nico-

tinic acid values in feedstuffs as given by Hale, Davis and Baldwin ('42).

The studies with laying hens (New Hampshire pullets) were conducted in conventional-type steel laying batteries. Artificial inseminations were performed twice weekly with the pooled semen of three New Hampshire roosters. Eggs were collected daily. Weekly settings of eggs were made and hatchability recorded.

RESULTS

In table 1 a summary is given of a number of experiments dealing with the effect of the addition of various levels of gelatin to ration 108GN, with and without nicotinic acid, on mortality, growth, blacktongue, perosis, and efficiency of feed utilization. Results obtained with the presence of corn are also given.

As the level of gelatin (which supplies sufficient arginine and glycine for the chicken when fed at a level of 10%) was increased in the ration above the 5% level, an actual depression in growth rate occurred (compare groups 1, 3, 6, and 13). Growth depression was accompanied by increased mortality, perosis, and blacktongue. Other deficiency symptoms such as poor feathering, diarrhea, excessive amount of mucous in the mouth, unkempt appearance of the head, and accumulation of feed under the tongue ("food canker") were noted in a majority of the chicks. Some of these latter "symptoms" may have been due to the physical character of the ration (i.e., the presence of gelatin causes the ration to become sticky when moistened). The growth depression was most marked with the highest levels of gelatin fed but was overcome by the addition of nicotinic acid or tryptophane to the ration. Lower amounts of tryptophane were not as effective (Briggs, '45).

It is evident, as would be expected, that sufficient arginine and glycine (supplied by gelatin) must be present before nicotinic acid can give maximum growth (compare group 2 with groups 17 and 18). The lack of perosis with 20% gelatin alone may be attributed to the very poor growth rate. Higher

Effect of gelatin and corn on growth, blacktongue, perosis, efficiency, and nicotinic acid requirement of chicks.

GROUP	SUPPLEMENT TO BASAL RATION 108Gm (WITH NO GELATIN)	NO. CHICKS STARTED	NO. DEATH 4 WKS.	AVERAGE WEIGHT AT 4 WKS.	PER CENT WITH BLACK- TONGUE	PER CENT WITH PEROSIS	EFFICIENCY ¹
				gm			
1	None	12	1	88	8	25	0.350
2	5 mg nicotinic acid/100 gm	12	2	101	0	0	0.275
3	5% gelatin	35	1	247	43	20	0.505
4	5% gelatin + 1 mg nicotinic acid/100 gm	6	0	283	0	17	0.505
5	5% gelatin + 5 mg nicotinic acid/100 gm	18	0	298	0	0	0.521
6	10% gelatin	72	12	138	89	64	0.358
7	10% gelatin + 1 mg nicotinic acid/100 gm	6	0	237	67	50	0.546
8	10% gelatin + 2 mg nicotinic acid/100 gm	12	1	313	0	42	0.526
9	10% gelatin + 3 mg nicotinic acid/100 gm	6	0	299	0	67	0.547
10	10% gelatin + 5 mg nicotinic acid/100 gm	65	1	328	0	15	0.565
11	10% gelatin + 10 mg nicotinic acid/100 gm	12	1	333	0	8	0.512
12	10% gelatin + 0.2% dl-tryptophane	12	0	343	0	25	0.588
13	20% gelatin	6	5	75	83	0	0.148
14	20% gelatin + 5 mg nicotinic acid/100 gm	12	1	333	33	50	0.581
15	10% bone osscin	6	2	116	83	17	0.296
16	10% bone osscin + 5 mg nicotinic acid/100 gm	12	0	311	0	0	0.600
17	0.8% l (+) arginine + 2.5% glycine	30	1	258	37	30	0.517
18	As group 17 + 5 mg nicotinic acid/100 gm	6	0	296	0	0	0.445
19	As group 17 + 1% alanine	6	0	208	0	83	0.534
Following groups contain corn in place of Cerelease:							
20	10% gelatin	12	2	130	92	75	0.370
21	10% gelatin + 1 mg nicotinic acid/100 gm	6	0	248	50	50	0.530
22	10% gelatin + 2 mg nicotinic acid/100 gm	6	0	259	0	50	0.591
23	10% gelatin + 5 mg nicotinic acid/100 gm	6	0	373	0	33	0.647
24	10% gelatin + 10 mg nicotinic acid/100 gm	6	0	364	0	0	0.619
25	10% gelatin + 0.2% dl-tryptophane	12	0	306	0	60	0.599

¹ Total weight gained

Total feed consumed

levels of gelatin caused the ration to become too gummy when eaten by the chicks.

Bone ossein, from which gelatin is derived, caused a similar depression of growth in the absence of nicotinic acid.

When an amount of arginine and glycine equal to that found in 10% of gelatin was fed, near maximum growth occurred. This was improved further, however, by the addition of nicotinic acid to the ration. The addition of alanine to the combination of arginine and glycine definitely depressed the growth rate and gave a high incidence of perosis (group 19, table 1).

The feeding of large amounts of corn with gelatin gave results similar to those with gelatin alone. Apparently the action of the corn and gelatin was not additive with 10% of gelatin in the ration. The addition of nicotinic acid to the corn rations resulted in an extremely fast rate of growth for this species (appreciably greater growth than when nicotinic acid was added to the Cerelese rations). This suggests that corn contains an unidentified growth-promoting factor or factors not present in the basal ration. Tryptophane appeared to be somewhat less effective when added to the corn diet than when added to the Cerelese diet (compare groups 12 and 25).

It is evident, from data presented in table 1, that the amount of nicotinic acid which was necessary for maximum growth varied with the amount of gelatin or tryptophane fed. For example, 1 mg of nicotinic acid resulted in better growth and less blacktongue and perosis with the presence of 5% of gelatin than with 10% of gelatin (compare group 4 with group 7). No nicotinic acid was needed for growth when ample tryptophane was present although some perosis did occur. Thus, the chicken may require anywhere from little or no nicotinic acid in a diet to as high as at least 5 to 10 mg per 100 gm of ration. The amount required by New Hampshire chicks on this ration (a minimum of approximately 5 mg per 100 gm) appears to be considerably higher than the amount set by Briggs et al. ('42) for the White Leghorn chick (1.8 mg per 100 gm of a similar ration containing 10% of gelatin) and

is similar to the amount required by the turkey under such a regime (Briggs, '46).

*Method of assay of factors responsible for
depressing action*

Because fairly good growth was obtained by the inclusion of only 5% of gelatin in the ration whereas higher levels of gelatin caused a marked depression in growth, a means of assay could be devised for the routine determination of the depressing action of various supplements. The assay was based upon the depressing effect of the supplements when added to the basal ration 108GN plus 5% of gelatin.

The results of such an assay are summarized in table 2. The addition of high levels of gelatin (groups 2 and 3) and ossein (group 4) to the ration had severe depressing effects. (This character of these proteins was later utilized in producing a nicotinic acid deficiency in chicks receiving practical rations and in hens). Casein and soybean oil meal, as well as oats and wheat (groups 5 to 8), had no depressing effect.

By comparing the activities of corn, corn gluten meal, and zein (groups 9 to 13), it is evident that the depressing action of corn is due to its protein content, as Krehl, Sarma and Elvehjem ('46) have likewise reported. The depressing action of corn and corn gluten meal was again prevented by the presence of nicotinic acid. It is very interesting to note that zein was only about one-third as depressing as gelatin (see groups 2, 3, and 11). This indicated that more is probably involved in this problem than just the feeding of a tryptophane-low protein itself.

Since such a marked depression was obtained with gelatin, and since it is known that certain of the amino acids in gelatin have a detrimental effect when added to certain rations (see review by Hier, Graham and Klein, '44; and also Patton, '39), several amino acids were fed in combination with 5% of gelatin (groups 14 to 20, table 2). Again alanine had a definite depressing action as did the combination of arginine and glycine. The depressing action of these amino acids was ap-

tinic acid and with casein showed that the protein quality in ration 2 was poor or that certain other nutrients were lacking.

Studies with laying hens receiving purified diets

In preliminary work ration 104N,² similar to the basal purified ration 108GN but with 5% of gelatin, was fed to four laying New Hampshire pullets. Four additional pullets were fed ration 104N supplemented with 20 mg of nicotinic acid per 100 gm of ration. No differences in egg production, body weight, or hatchability were noted after a 3 months' feeding trial.

When it was found in the work with chicks that greater amounts of gelatin or bone ossein than 5% were necessary to give a depressing action in the absence of nicotinic acid, eight more New Hampshire pullets were placed on experiment and were divided into two groups of four hens each, with and without nicotinic acid. They were fed a basal ration similar to ration 104N but with a total of 20% of ossein. Corn starch was used instead of Cerelese. Ossein was chosen because it did not cause the ration to be sticky when moistened, as did gelatin.

The four hens given the basal ration without nicotinic acid (group A) began to lose weight rapidly and showed a loss of egg production as well as a marked drop in hatchability (see table 4 and fig. 1). Many of the eggs were soft-shelled. The hens in group B, which were given 50 mg of nicotinic acid per 100 gm of ration, maintained their weight well and had normal egg production and hatchability.

After a period of 6 weeks, when all the hens in the deficient group had lost considerable weight and one had become quite moribund, the rations were reversed. Within a few days hens in group A began to gain weight and continued to gain throughout the experiment, with the exception of the moribund hen which died. Egg production and hatchability increased

² Ration 104N contained only 15% of casein, 5% of gelatin, and an increased amount of vitamins A and D by capsule (7 drops) weekly. Oyster shell was fed ad libitum.

TABLE 4

The effect of a nicotinic acid deficiency in laying hens on egg production and hatchability.

GROUP A NO NICOTINIC ACID			GROUP B WITH NICOTINIC ACID		
Weeks on experimental diets	Eggs/hen/day	Per cent hatchability	Weeks on experimental diets	Eggs/hen/day	Per cent hatchability
1	0.392	100	1	0.678	72.7
2	0.385	50	2	0.500	55.6
3	0.250	0	3	0.642	90.0
4	0.211	0	4	0.714	87.5
5	0.285	0	5	0.678	72.7
6	0.107	0	6	0.642	81.8
Diets reversed					
7	0.035	0	7	0.642	62.5
8	0.211	80	8	0.535	67.0
9	0.250	67	9	0.500	00
10	0.240	100	10	0.392	28.6
11	0.190	100	11	0.250	33.3
12	0.238		12	0.250	
13	0.333		13	0.285	

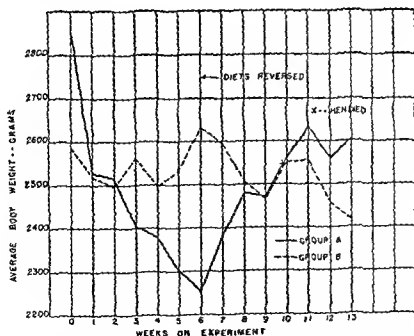


Fig. 1 Growth results with hens receiving high amounts of ossein. Group A, no nicotinic acid (first 6 weeks); Group B, with nicotinic acid (first 6 weeks). Diets reversed at 6 weeks.

markedly. The former positive control birds (group B), which were now given the deficient ration, lost weight and had a marked drop in egg production. The experiments were discontinued when ground ossein no longer was available.

Although a relatively small number of hens was used, the results showed quite conclusively, and for the first time, that hens under certain conditions require nicotinic acid in the diet for proper weight maintenance, egg production, and hatchability. No "blacktongue" symptoms were noted in the hens.

DISCUSSION

The reason for the depressing effect of gelatin and similar proteins low in tryptophane, and its counteraction with nicotinic acid, has not been accurately determined as yet. The data presented in this paper indicate that certain of the amino acids present in the proteins are at least partially responsible for this detrimental action. The amino acids arginine, glycine, and alanine account for some of the depressing action of gelatin. It is possible that nicotinic acid is necessary for the metabolism of excess amino groups. This view is strengthened by the work of Dann ('46b) who found that 2% of nicotinamide in a practical diet depressed the growth rate of chicks considerably whereas this same level of nicotinic acid did not.

Woolley ('46), in work with mice, has recently published evidence of a potent "pellagragenic" agent, in corn, which has been considerably concentrated. It is soluble in a mixture of chloroform and sodium hydroxide. No direct evidence for the existence or non-existence of such a factor in gelatin or corn, other than the amino acids themselves, has been found in our studies.

No reason has been demonstrated in this work as to why it is possible to replace nicotinic acid in the ration with tryptophane. However, the recent work of Rosen, Huff and Perlzweig ('46) and Singal, Briggs, Sydenstricker and Littlejohn ('46) with rats indicates that tryptophane may be an important precursor of nicotinic acid synthesis. It is well known that tryptophane can give rise to kynurenic acid, containing

a pyridine ring, which indicates that such synthesis may be possible. In our studies, the addition of sufficient nicotinic acid to the basal ration was always as effective as feeding much larger amounts of tryptophane. Thus, we have no evidence that tryptophane is being synthesized in the intestinal tract due to the presence of nicotinic acid, as suggested by Krehl, Sarma, Teply and Elvehjem ('46). Also, we feel, in the case of the chick at least, that most of the synthesis of nicotinic acid is within the body tissues rather than in the intestinal tract. Briggs, Luckey, Teply, Elvehjem and Hart ('43) showed that only a small portion of the chick's nicotinic acid requirement on such diets is synthesized within the intestinal tract. The fact that the depressing action is obtained on two widely different rations is further evidence of synthesis within the tissues of the chicken.

Since the tryptophane requirement of chickens is dependent upon the amount of nicotinic acid in the ration (and vice versa), it is difficult to attempt to set the dietary requirements for nicotinic acid or tryptophane unless the composition of the diet is considered. It is clear from our results that the amount of tryptophane supplied by 18% of casein (approximately 0.22 gm per 100 gm according to Kratzer, '44) is sufficient when ample nicotinic acid is present. The requirement of tryptophane for chicks, as determined by Grau and Almquist ('44), was 0.25% of the diet.

Berry, Carriek, Roberts and Hauge ('43), and Gericke ('44), have given evidence that certain practical poultry rations may be deficient in nicotinic acid. Richardson, Hogan and Kempster ('45) noted an actual increase in the incidence of perosis when nicotinic acid was omitted from a practical ration "diluted" with a synthetic ration. Briggs et al. ('43) previously had observed perosis in nicotinic acid-deficient chicks.

The results given in this study show that a nicotinic acid deficiency may occur in chickens under practical conditions if the protein (amino acid) content is unbalanced. It is interesting to note in this respect that Prange, Hauge and Carriek

('27) reported that gelatin caused a depression in growth rates of chickens when added to a practical ration containing large amounts of corn and meat meal. Also, McGinnis ('46) and Lucas, Norris and Heuser ('46), working with turkeys and chickens, respectively, have shown that gelatin increases the incidence of perosis when added to certain diets. Jukes ('41) reported that gelatin, when added to certain rations, increased the incidence of perosis. Perosis was prevented entirely by the addition of choline, however. It is interesting to note further that Massengale ('29) reported that high levels of meat scraps (which contain considerable gelatin) in rations for poultry resulted in poor growth and in a condition similar to perosis. The addition of yeast or milk corrected the condition. It is quite possible that an unrecognized nicotinic acid deficiency was produced in all these cases although nicotinic acid was not considered. The diets appear to have less nicotinic acid in them than the minimum amount necessary under extreme conditions.

Considerable work has been done with other animals in respect to an inhibitory action of gelatin on growth. Again it is possible that a nicotinic acid deficiency was involved. Hier et al. ('44) have noted an inhibitory action of gelatin in certain rations for rats, as well as Jackson, Sommer and Rose ('28) who reviewed the older literature on this subject. References to work with humans in this respect may be found in a report by Robschey-Robbins, Miller and Whipple ('44).

It is well known that an excessive amount of corn in rations for chickens may cause growth depression or other difficulties. Taylor, Lerner and De Ome ('44) have noted an increase in laying hen mortality due to the presence of high amounts of corn in the ration. These authors reviewed previous work with chickens in this respect. In light of the present study and the recent work by Sarma and Elvehjem ('46) on the influence of corn on the nicotinic acid requirement of chickens, an adequate supply of nicotinic acid in the diet in future work of this type would be important.

SUMMARY

The nicotinic acid requirement of chickens was influenced by the protein content of the ration. The presence of an excessive amount of a protein low in tryptophane (e.g., gelatin or zein) in purified, nicotinic acid-low rations depressed the growth rate of young chickens and increased the severity of nicotinic acid deficiency symptoms (blacktongue, perosis, etc.). Gelatin was about three times more active than zein in this respect. The depressing effect was overcome by the addition of nicotinic acid or tryptophane to the ration. A dietary source of nicotinic acid was not apparently required if sufficient tryptophane was present in the ration.

The New Hampshire chick may require at least 5 mg of nicotinic acid per 100 gm of diet under certain extreme conditions.

By the addition of bone ossein to a highly purified ration, it was possible to obtain a nicotinic acid deficiency in laying hens with a resulting drop in egg production, body weight, and hatchability. By the same procedure a nicotinic acid deficiency was produced in young chickens receiving a practical ration high in corn and corn products.

A definite depression in growth rate and an increase in nicotinic acid deficiency symptoms were obtained by feeding certain combinations of arginine, glycine, and alanine. Thus, at least part of the depressing action of gelatin may be accounted for by its amino acid content.

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THE UTILIZATION OF IRON FROM DIFFERENT FOODS BY NORMAL YOUNG RATS¹

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THREE FIGURES

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Most of the information concerning the utilization of the iron of different foods has been obtained by studies with animals rendered anemic by hemorrhage, exclusive milk feeding, or other means. In young rats made anemic by exclusive milk feeding, differences in the ability of different foods to regenerate hemoglobin have been observed. Most iron salts are readily utilized for hemoglobin regeneration, providing sufficient copper is present. Experiments by Rose and Vahlteich ('32) and Free and Bing ('36) have indicated that the iron of whole wheat is about as well utilized by the rat as that of ferrie chloride, although there has been controversy on this point, apparently arising from a difference in the method of feeding employed (Nakamura and Mitchell, '43). The work of Rose and Kung ('32) and Rose, Vahlteich and MacLeod ('34) has shown that the iron of beef is not as well utilized by the rat as that of whole wheat; also that the iron of beef liver is more available than that of the muscle.

Ascham, Speirs and Maddox ('38) and Sheets and Ward ('40) demonstrated that the iron of leafy vegetables is less available for the rat than that of ferrie chloride. That the

¹The data in this paper were taken from a dissertation submitted by Orrea Florence Pye in partial fulfillment of the requirements for the degree of doctor of philosophy under the Joint Committee on Graduate Instruction, Columbia University.

iron of egg yolk is well utilized if there is an adequate supply of copper was indicated by the work of Rose, Vahlteich and MacLeod ('34) and Sherman, Hart and Elvehjem ('34).

Since so much of our information concerning the availability of iron from different foods has been acquired under the abnormal conditions of experimental anemia where factors not applicable to the normal situation might conceivably prove a complication, it seemed worthwhile to study the utilization of iron in normal young rats under dietary conditions approximating normal as closely as possible. The present investigation was designed, therefore, to follow the hemoglobin values of and determine the iron stored by normal young rats given, for a 6-week experimental period, supplies of iron which were within normal dietary limits. Since interest was centered on maintaining as nearly as possible a normal dietary situation, no attempt was made to compensate for the varying effect of different food supplements on the growth rate of the animals, but in order to counteract the effect of differences in body size, iron dosage was adjusted to body weight.

EXPERIMENTAL PROCEDURES

Two series of experimental diets were used. In Series I equal amounts of iron were supplied by different supplements, while in Series II differing levels of iron were fed.

Male albino rats 3 weeks of age were used, all from a stock colony reared on a diet of one-third whole milk powder, two-thirds whole wheat flour, and sodium chloride equal to 2% of the weight of the flour. Initial, weekly, and final hemoglobin determinations were made according to the Newcomer method on freely flowing blood obtained by cutting the tip of the tail of the animal. Several littermates were killed at 3 weeks of age to determine average iron content at the beginning of the 6-week experimental period. At the end of the period all animals were analyzed for iron content. All food materials were also analyzed. The method of analysis for iron was in general that employed by Rose and Hubbell ('38), Kunerth ('40), and Houghton ('42) in this laboratory. A method of

wet ashing with sulfuric and nitric acids suggested by Roberts, Beardsley and Taylor ('40) was adapted for use with a few of the foods analyzed.

Series I

Two basal rations were used. Basal diet A was one used successfully by Kumerth ('40) for rendering rats anemic. Since this diet with certain food supplements proved to be somewhat laxative, basal diet B (consisting of dried whole milk with daily administration of thiamine chloride and pyridoxine) was substituted in these cases. Eight food supplements (beef muscle, uncooked and cooked kale, uncooked and cooked spinach, whole wheat flour, beef liver, and egg yolk) and an iron salt (ferric chloride) were given in amounts to furnish the minimum quantity of iron needed by the young rat, 0.002 mg iron per gm of body weight daily (Rose and Hubbell, '38). Copper sulfate was added when the iron salt or egg yolk was given. The iron supplements were prepared at the beginning of the experimental period and all precautions taken to avoid accidental inclusion of iron in drying and pulverizing the foods. The beef muscle and beef liver were not cooked before drying but the egg yolk was. The animals were weighed weekly and the amount of iron required for each animal for the coming week computed according to this weight.

Series II

Four diets were used in Series II. Diet I consisted of one-third whole milk powder, two-thirds whole wheat flour, and sodium chloride equal to 2% of the total weight of the flour. Diet II was like Diet I except that half of the whole wheat flour was replaced by unenriched patent flour. To compensate for some of the losses in milling the flour, supplements were given as follows: 0.8 μ g manganese and 0.3 μ g copper per gm of rat per day, and 25 μ g thiamine chloride, 2 μ g riboflavin, and 4 μ g pyridoxine per animal per day. Diet III had all of the whole wheat flour of Diet I replaced by unenriched

patent flour with mineral and vitamin supplements double those of Diet II. Diet IV was in all respects like Diet III except that sufficient ferric chloride was added to make the total iron content equal to that of Diet I.

RESULTS AND DISCUSSION

Series I

Average results are shown in table 1. The average weekly hemoglobin values of the rats receiving Diet A plus supplements are shown in figure 1. The average final hemoglobin level

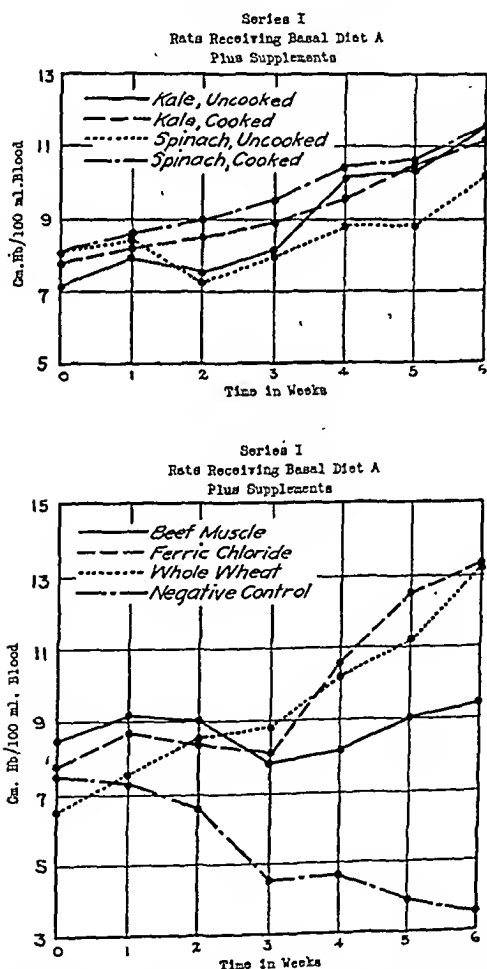


Fig. 1 Average weekly hemoglobin values of Series I rats receiving basal diet A plus supplements.

TABLE 1

Average results in 6-week experimental period. (Range of values given in parentheses.)

NO OF ANIMALS	SUPPLEMENT ON DIET	WEIGHT		HEMOGLOBIN PER 100 ML. OF BLOOD			IRON INTAKE		IRON CONTENT ¹ PER GR. OF RAT	RETENTION ¹
		Initial	Final	Initial	Final	Gain	From supplement	From basal diet		
		gm	gm	gm	gm	gm	mg	mg		%
Series I. Animals receiving a basal diet plus different supplements										
<i>Diet A</i>										
10	Beef muscle	39	184	8.5 (7.1-9.6)	9.4 (8.4-10.5)	0.9	8.51	1.01	0.022 (.015-.026)	32
5	Kale, uncooked	45	140	7.2 (6.5-8.0)	11.5 (10.2-13.0)	4.3	6.82	1.03	0.023 (.019-.029)	28
7	Kale, cooked	37	133	7.7 (7.0-8.2)	11.3 (9.8-13.3)	3.6	6.01	1.03	0.024 (.017-.027)	29
10	Spinach, uncooked	42	154	8.1 (6.9-10.1)	10.1 (8.7-12.1)	2.0	7.52	1.10	0.021 (.016-.026)	20
7	Spinach, cooked	37	135	8.0 (7.2-8.8)	11.5 (10.5-12.3)	3.5	6.54	1.08	0.026 (.021-.033)	31
5	Whole wheat flour	40	140	6.5 (6.2-7.0)	13.3 (12.8-13.0)	6.8	7.10	0.65	0.033 (.029-.038)	42
12	Ferrie chlorido + copper sulfato	38	172	7.8 (6.2-8.9)	13.4 (11.6-14.9)	5.6	8.18	1.42	0.031 (.023-.030)	42
2	Negative control	40	103	7.4	3.4	-4.0		1.01	0.012	
<i>Diet B</i>										
6	Beef liver	38	193	9.1 (8.5-10.2)	13.5 (12.0-14.4)	4.4	8.96	1.36	0.029 (.022-.038)	42
6	Egg yolk + copper sulfato	40	170	7.6 (7.0-8.2)	12.1 (10.5-13.2)	4.5	8.31	1.27	0.025 (.018-.030)	33
8	Ferrie chlorido + copper sulfato	37	152	10.0 (9.3-10.5)	14.6 (14.0-15.2)	4.6	7.27	1.35	0.033 (.029-.039)	46
3	Negative control	39	115	9.3	3.8	-5.5		0.98	0.013	
Series II. Animals receiving diets varying in iron content										
								Total from diet		
11	Diet I	37	165	7.8 (6.2-9.6)	14.7 (14.0-15.3)	6.9		9.86	0.040 (.030-.047)	54
12	Diet II	37	143	7.6 (6.2-8.9)	11.5 (10.2-12.5)	3.9		5.60	0.026 (.020-.031)	50
12	Diet III	39	134	7.4 (6.2-8.5)	6.6 (5.1-7.9)	-0.8		2.61	0.018 (.014-.021)	46
12	Diet IV	39	166	8.2 (6.7-8.9)	13.9 (11.8-15.1)	5.7		9.14	0.037 (.029-.043)	49

¹ Based on net weight, that is, final live weight minus weight of intestinal tract.

attained by the animals receiving whole wheat flour (13.3 gm per 100 ml of blood) was practically identical with that reached by the rats given ferric chloride (13.4 gm). The rats receiving whole wheat flour made a greater gain in hemoglobin in the 6 weeks. This may have been due to the lower initial hemoglobin level in these rats, for in general, the animals with lower initial levels made greater hemoglobin gains in the 6-week period.

The iron content of the animals on whole wheat flour was very similar to that of the rats given ferric chloride, averaging 0.033 mg and 0.031 mg iron per gm of rat, respectively. In both cases the body iron was appreciably higher than in the 21-day-old animals, which averaged 0.022 mg iron per gm of body weight (range, 0.019–0.025 mg).

Of all the supplements fed, dried beef muscle was the least satisfactory in building hemoglobin, resulting in an average final level of 9.4 gm per 100 ml of blood, only slightly higher than the average initial level of 8.5 mg. The body iron per gm of rat of the animals fed the dried beef muscle did not increase in the 6-week period.

The average final hemoglobin values of the animals fed uncooked kale, cooked kale, uncooked spinach, and cooked spinach were, respectively, 11.5 gm, 11.3 gm, 10.1 gm, and 11.5 gm per 100 ml of blood. The average values for iron content were, in the same order, 0.023 mg, 0.024 mg, 0.021 mg, and 0.026 mg per gm of body weight. Ascham, Speirs and Maddox ('38) found slightly better hemoglobin response to spinach than to kale in rats rendered anemic. In this study the hemoglobin-building value of the iron of kale and spinach, cooked or uncooked, appears to be practically the same and in no case equal to that of the iron of whole wheat flour, ferric chloride, or beef liver.

The average weekly hemoglobin values of rats receiving Diet B plus supplements are shown in figure 2.

The animals fed dried beef liver attained an average final hemoglobin level of 13.5 gm per 100 ml of blood and an average iron content of 0.029 mg per gm, lower than the value of

0.033 mg per gm found in the rats given ferric chloride. The dried beef liver, as prepared in this study, while not as effective either in the production of hemoglobin or the deposit of iron in the tissues as the iron salt, still appeared to be well utilized by the normal young rat. In the experiments of Rose and Kung ('32) the iron of liver appeared to be effective in the regeneration of hemoglobin although not as effective as the iron of whole wheat. In this study the amount of iron given was adjusted to body weight. Therefore the rats

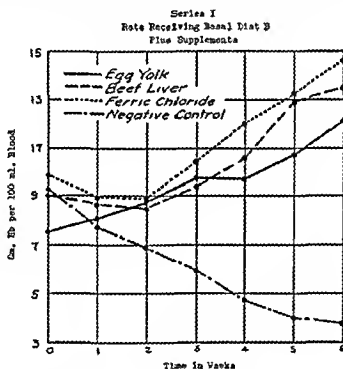


Fig. 2 Average weekly hemoglobin values of Series I rats receiving basal diet B plus supplements.

which were receiving the liver supplement and making greater weight gains received a larger supply of iron during the 6 weeks (10.34 mg) than the slower-growing animals on the iron salt supplement which obtained only 8.62 mg. If the iron ingested had been kept identical for both lots of animals, irrespective of weight, those on the liver supplement would have been at a disadvantage because they tended to grow faster.

The animals fed egg yolk plus 0.2 μ g copper daily per gm of body weight attained a final level of only 12.1 gm hemo-

globin per 100 ml of blood. It may be that not enough additional copper was provided, as insoluble copper salts tend to form when egg yolk is fed. Copper may have been the limiting factor in hemoglobin formation but it could not bring about the low iron content per gm (0.025 mg) in these animals as it has been demonstrated that iron is stored in the absence of copper. Tompsett ('40) in an experiment in which mice were fed egg yolk as a source of iron found poor iron storage which he attributed to the interference of phosphatides and phosphoproteins with the absorption of iron.

Series II

Average results are shown in table 1 and average weekly hemoglobin values of the rats in this series in figure 3.

The iron content per 100 gm of the four diets was found upon analysis to be as follows: Diet I, 2.63 mg; Diet II, 1.88 mg; Diet III, 0.97 mg; Diet IV, 2.54 mg. The average

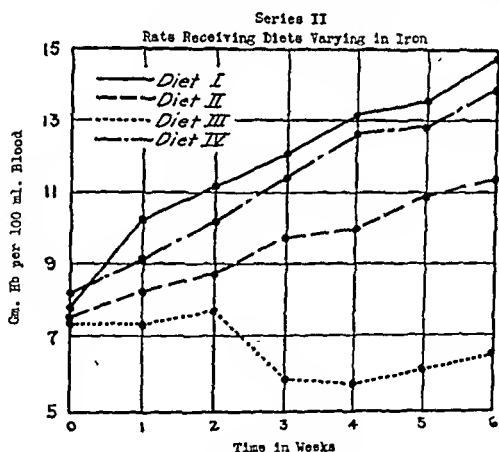


Fig. 3 Average weekly hemoglobin values of Series II rats receiving diets varying in iron content.

1.88 mg; Diet III, 0.97 mg; Diet IV, 2.54 mg. The average final hemoglobin levels per 100 ml of blood of the animals on the four diets were as follows: Diet I, 14.7 gm; Diet II, 11.5 gm; Diet III, 6.6 gm; Diet IV, 13.9 gm. Diet II did not provide sufficient iron to bring the hemoglobin up to the

normal level in the 6-week period. On Diet III the majority of the animals not only were unable to maintain the hemoglobin at the 21-day-old level, but showed a small decrease. On Diet I the hemoglobin values were all normal for 9-week-old rats. On Diet IV a few individual animals fell below the normal range.

Of the four diets, average iron content values per gm of rat were as follows: Diet I, 0.040 mg; Diet II, 0.026 mg; Diet III, 0.018 mg; Diet IV, 0.037 mg. Iron stores as well as hemoglobin values varied according to the quantity of iron ingested, although not in direct proportion.

Retention of iron. Percentage retention of iron on the different supplements and diets was computed according to the following formula: $\frac{\text{iron retained}}{\text{total iron ingested}} \times 100$. Iron retained was taken to be the difference between the average iron content (based on the final live weight of the animals minus the weight of the intestinal tract) at 3 weeks of age and after 6 weeks on the experimental diet.

Iron retention on Diet A plus supplements varied from 26% for uncooked spinach to 42% for both whole wheat flour and ferrie chloride. When the percentage retention of the ferrie chloride on the two basal diets is compared, Diet B has a slight, although probably not a significant advantage—46% versus 42% on Diet A.

The percentage retention of iron was higher on the diets in Series II than on those in Series I. The percentage retention in Series II was as follows: Diet I, 54; Diet II, 50; Diet III, 46; and on Diet IV, 49. The highest retention in Series I was 46% on Diet B plus ferrie chloride. The average level of iron intake on the diets in Series I was between that on Diets I and II in Series II. However, the animals on Diets I and II ingested a larger quantity of the total iron in the early part of the experimental period and a smaller quantity in the later part than the animals on the diets in Series I. Other variables, such as amino acids, copper, phytin, some of the vitamins, may also have affected the utilization of the iron in the different diets.

SUMMARY AND CONCLUSIONS

I. When normal young rats were fed daily 0.002 mg iron per gm of body weight from different sources and hemoglobin formation and iron retention in a 6-week period were used as the criteria of "availability," it was found that:

1. The iron of whole wheat flour was about equal in availability to that of ferric chloride.

2. The iron of beef liver was almost but not quite as available as that of ferric chloride.

3. The iron of the other food materials tested (beef muscle, egg yolk, uncooked and cooked kale, and uncooked and cooked spinach) was less available than that of beef liver; the differences among these supplements were small.

4. In general the order of "iron availability" of these different foods was the same as that observed in studies of hemoglobin regeneration in anemic rats, but the differences were smaller.

II. When normal young rats were given diets containing readily available sources of iron (I) whole wheat flour, (II) half whole wheat, half unenriched patent flour, (III) unenriched patent flour, (IV) unenriched patent flour plus ferric chloride, for a 6-week period, it was found that:

1. Hemoglobin production and iron storage were improved when the level of intake of iron averaged slightly more than 0.002 mg per gm of body weight daily (Diet I)..

2. Levels of iron averaging lower than 0.002 mg iron per gm of body weight daily were insufficient to bring about good hemoglobin production; a level slightly lower resulted in a small hemoglobin gain (Diet II) but when the level of iron averaged below 0.001 mg (Diet III) no gain was observed in 6 weeks.

3. Levels of iron averaging lower than 0.002 mg iron per gm of body weight were insufficient to bring about much increase in the iron content per gm of rat in 6 weeks; a slightly lower level resulted in a slight increase (Diet II), but when the level of iron averaged below 0.001 mg (Diet III) a decrease was observed.

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The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in Chicago, May 18-22, 1947. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1947. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

GENEVIEVE STEARNS,
*College of Medicine,
State University of Iowa,
Iowa City, Iowa*

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY "B-COMPLEX" AWARD

Nominations are solicited for the 1947 Award of \$1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute, May 18-22, 1947.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1 to December 31 the most meritorious scientific report dealing with the field of the "B-complex" vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1946 must be in the hands of the Chairman of the Nominating Committee by January 15, 1947. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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CHAIRMAN, NOMINATING COMMITTEE

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